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RETICULOCYTOSIS IN GUINEA-PIGS IN RELATION TO THE STANDARDIZATION OF LIVER-EXTRACT.

GUNNAR ÅGREN AND TORBJÖRN CASPERSSON.

From the Department of Biochemistry of the Caroline Institute, Stockholm.

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FOR the standardization of the anti-anæmic principles in the liver the degree of reticulocytosis produced in experimental animals has recently been used as a criterion. Most of the methods employed have been found to be unreliable, and it appears that the guinea-pig test (Jacobson, 1934, 1935) is the only one that has been used as an indicator. Jacobson's method is as follows: Guinea-pigs are injected with small doses of liver-extract of known activity. A certain percentage react with a temporary reticulocytosis. These animals are then used for testing the preparations to be investigated. The proportion of reticulocytes that is interpreted as a positive result is 2–4 p.c. of the total number of red blood-corpuscles. The dose required is exceedingly small, a positive reaction is obtained with an extract from approximately 0·2 mg. of fresh pig's liver.

As this method has been much used, and as no statement is to be found in the literature about reticulocytosis in normal guinea-pigs, we believe that the following series of experiments will be of interest.

Procedure.—Fifty guinea-pigs of the same size, all bred under the same conditions, were used. On the same day 25 of them were injected with 1/2 c.c. liver extract (= 10 g. fresh liver) that had been proved to be potent on human patients. Every second day the percentage of reticulocytes in all 50 animals was determined. The blood sample (0·005 ml.) for the counting of the reticulocytes was taken from a small cut in the ear, and stained by Ederle's method. The advantages of this method of staining with a concentrated solution of brilliant cresyl blue (1 p.c.) have been pointed out by Gurd (1935).

Every precaution was taken to avoid a loss of blood when getting the samples, and these never exceeded 10–20 c.mm.

Result.—The estimation of the reticulocytosis showed that this is subject to much individual variation. Periods with a reticulocyte percentage of about 0·25 to 1 are often followed by periods in which their frequency is increased to about 2–4 p.c. It is impossible to determine from our results how often such peaks occur. During the three weeks of our observations increased reticulocytosis was seen in most of the animals. In some cases it started quite irregularly throughout the period of observation; in others only the decreasing part of the curve was manifested. In a few animals, who behaved in the last-mentioned manner and which thus seemed to have just completed the regeneration period, the beginning of a new discharge could be observed after the lapse of about two weeks. These peaks appeared with exactly the same frequency in the animals injected with liver-extracts as in the controls (see table). These facts show that the reticulocytosis when being judged as a specific effect must be of an entirely different order. No effect attributable to the liver treatment could be observed in our experiments, though we used a dose of extract which was enormous in comparison with those mentioned by other authors.

TABLE.—*Percentage of Reticulocytes: Average of the Two Series* (25 *Animals in each*).

Day.	1.	3.	5.	7.	9.	11.	13.	15.
Untreated . .	0·61	0·67	0·86	1·47	1·29	1·15	1·16	1·38
Liver-treated . .	0·78	0·76	0·79	1·65	1·28	1·53	1·34	1·39

SUMMARY.

The percentage of reticulocytes in the blood of guinea-pigs is normally subjected to considerable variations. In a series of 25 guinea-pigs treated with a purified liver preparation of proved activity, these variations occurred with statistically the same frequency and character as in the untreated controls.

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THE MECHANISM OF IMMUNITY IN RIFT VALLEY FEVER.

G. M. FINDLAY.

Wellcome Bureau of Scientific Research, London.

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OF the many problems raised by the study of viruses, one of the most interesting is the question whether *in vitro* there is any direct combination between a virus and its specific immune serum. Many observers believe that no such combination can be demonstrated, as (i) an apparently neutral mixture of virus and immune body can be rendered active—"dissociated"—by simple dilution, and (ii) an apparently neutral mixture, inactive when inoculated by one route, may be active when inoculated by another. On the other hand, there have not been lacking other experiments which suggest that under certain conditions a combination between virus and immune serum may occur *in vitro*.

It is not proposed here to review the many experiments already carried out on this problem; they are fully discussed by workers such as Sabin (1935) and Goyal (1935). A study of much of the evidence does, however, suggest that in many cases conclusions have been drawn from observations on a somewhat limited range of dilutions of virus and immune serum, the limitation being due for the most part to the difficulties in employing large numbers of animals for a single experiment.

In the present communication investigations are described on the interaction *in vitro* of Rift Valley fever virus and its immune serum. The virus of Rift Valley fever has certain advantages which make it of value for experiments of this nature. The virus has now been maintained under laboratory conditions in mice for nearly five years, and in this time has maintained a constantly high pathogenicity for these rodents, since an intraperitoneal injection of 0·2 c.c. of a liver suspension diluted 10^{-9} or 10^{-10} is almost invariably fatal within a few days.

On the other hand, the virus of Rift Valley fever has the disadvantage of also possessing a high infectivity for man—a property that has not been lost by repeated passage in laboratory animals. As a consequence, however, a considerable supply of human immune serum has been available, and in these experiments pooled serum has been used from A. E. G., G. M. F. and I. A. H. Details of the acquirement of immunity some four and a half years ago by the first two have already been given (Findlay, 1932), while details of the third infection are briefly recorded by Kitchen (1935). Incidentally, it is here shown that immune bodies, once acquired, remain in the circulation for some years after infection. Rift Valley fever is thus similar to yellow fever, though it differs from dengue and sandfly fever, in which diseases, after one attack, circulating immune bodies are not usually present for long periods. The virus

component used in all these experiments consisted of a suspension in saline of liver from mice inoculated 48 hours previously with Rift Valley fever virus. In all experiments equal quantities of immune serum and virus suspension were incubated together. The inoculum, unless otherwise stated, consisted of 0·4 c.c. of the serum-virus mixture injected intraperitoneally. Four mice were inoculated with each separate mixture. Previous observations on the mechanism of immunity in Rift Valley fever have been scanty. Occasional scattered experiments carried out during the past three years had shown that dissociation by dilution of an apparently neutral serum-virus mixture could readily be accomplished. In addition, Francis and Magill (1935) had shown that a serum-virus mixture, neutral on intraperitoneal inoculation, was active on intranasal instillation.

Experimental Results.

Experiment I.

An attempt was first made to determine whether dissociation by dilution occurs when a relatively large amount of immune serum is mixed with a comparatively small amount of virus.

A 10^{-1} dilution of infected mouse liver was diluted with saline to form dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}; equal parts of the original 10^{-1} dilution and the subsequent dilutions were then separately mixed with equal parts of undiluted immune Rift Valley serum and incubated at 37° C. for 4 hours. As a control, an equal part of undiluted normal human serum was mixed with each of the virus dilutions 10^{-1} to 10^{-5} and similarly incubated. After incubation, the mixtures of immune serum and virus dilutions were further diluted with saline to make four additional decimal dilutions, so that, for instance, from the original mixture of virus 10^{-5} and undiluted immune serum mixtures were made containing serum dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} with virus dilutions 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} respectively. The mixtures of undiluted normal serum and virus dilutions 10^{-1}, 10^{-2} and 10^{-3} were inoculated without further dilution, but the mixtures of undiluted normal serum and virus dilutions 10^{-4} and 10^{-5} were decimally diluted four times, so that the lowest dilution of virus was again 10^{-9}. Each serum-virus mixture was injected intraperitoneally into 4 mice. This procedure was followed in all the experiments to be described, except where otherwise stated.

The results are shown in Table I. It will be seen that the mixture of virus 10^{-9} + normal serum was active; on further dilution of the first three mixtures containing virus dilutions 10^{-1}, 10^{-2}, 10^{-3} and undiluted immune serum, the inactive mixtures were rendered fully active. On dilution of the mixture containing virus 10^{-4} and undiluted immune serum the activation was only partial, while on dilution of the mixture of virus 10^{-5} and undiluted immune serum there was no activation, although virus 10^{-9} was active, as shown by the result with normal serum.

Dissociation by dilution thus does not occur when virus is treated with an excess of immune serum. This result has been obtained on many other occasions; the same conclusions can be drawn from the figures given in Tables II and III.

Table I.*

Original virus dilution.	Normal serum (undiluted).	Dilution of serum (after incubation at 37° C. for 4 hours. Virus dilutions range from 10^{-2} to 10^{-9}).			
		10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.
10^{-1}	†2	..	..	..	..
	†2				
	†2				
	†2				
10^{-2}	†2	..	..	..	..
	†2				
	†2				
	†2				
10^{-3}	†2	..	..	..	..
	†2				
	†2				
	†2				
10^{-4}	†2	†2	†3	†3	†3
	†2	†2	†3	†3	†3
	†2	†2	†3	†3	†3
	†2	†2	†4	s	†6
10^{-5}	†2	†3	†3	†3	†3
	†2	†3	†3	†3	†4
	†2	†4	†4	†3	†5
	†2	†4	†5	†6	†6
	Immune serum (Undiluted).				
10^{-1}	†4	†3	†3	†3	†3
	†5	†3	†4	†3	†3
	s	†3	†5	†3	†3
	s	†3	†6	†4	†4
10^{-2}	s	†3	†3	†3	†3
	s	†4	†3	†3	†3
	s	†5	†3	†3	†3
	s	s	†6	†5	†3
10^{-3}	s	†5	†3	†3	†4
	s	†5	†3	†3	†7
	s	s	†4	†3	s
	s	s	†5	s	s
10^{-4}	s	†7	†4	†6	s
	s	s	†7	s	s
	s	s	†7	s	s
	s	s	†7	s	s
10^{-5}	s	s	s	s	s
	s	s	s	s	s
	s	s	s	s	s
	s	s	s	s	s

* In recording the various dilutions of serum and virus in the mixtures both in this and in succeeding Tables an intentional error has been made. A mixture consisting of equal parts of undiluted serum and virus 10^{-2} contains in reality a dilution of serum 1 in 2 and virus $0{\cdot}5 \times 10^{-2}$. On decimal dilution this becomes serum $0{\cdot}5 \times 10^{-1}$ and virus $0{\cdot}5 \times 10^{-3}$ and so on. In order not to overburden tables already complicated the 0·5 has been omitted throughout.

† = death: the subsequent figure indicates the number of days from inoculation to death.

s = survival.

Experiment II.

It having been shown that reactivation or dissociation by dilution did not occur when virus and an excess of serum were incubated together, an attempt was next made to determine whether a comparatively short exposure of virus to excess of serum would also inhibit reactivation by dilution.

Undiluted Rift Valley fever immune serum was mixed with equal volumes of suspensions of infected mouse liver diluted 10^{-4} and 10^{-5}; one half of each of these mixtures was kept at room temperature for 10 minutes. Then dilutions were made so that a series of mixtures was obtained containing virus dilutions 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}, together with immune serum dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively. The second halves of the original mixtures of undiluted immune serum and virus suspensions diluted 10^{-4} and 10^{-5} were incubated at 37° C. for 4 hours and then diluted as in the case of the mixtures kept at room temperature. As a control, a mixture of normal serum and a suspension of infected mouse liver 10^{-4} was in part diluted after 15 minutes at room temperature, in part after 4 hours at 37° C., so that the final dilution of virus was 10^{-9}, of serum 10^{-5}. The findings are shown in Table II, from which it will be seen that the results of 15 minutes and 4 hours' exposure of virus to excess of immune serum were similar in that they both inhibited reactivation on dilution.

Experiment III.

In order to demonstrate that the failure to obtain reactivation on dilution was specifically due to *excess* of immune serum acting on virus, the following experiment was carried out: Dilutions of infected mouse liver were made, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9}; equal amounts of undiluted immune serum and immune serum diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were also prepared. The undiluted immune serum was added to the virus dilution 10^{-4} and so on till the serum dilution 10^{-5} was added to the virus dilution 10^{-9}. These mixtures were then incubated at 37° C. for 4 hours. For the same time, equal quantities of undiluted immune serum and virus dilution 10^{-4} were incubated together, and after incubation, were decimally diluted to a final concentration of virus 10^{-9} and serum 10^{-5}.

As a control, undiluted serum was mixed with an equal quantity of virus dilution 10^{-4}, dilutions of serum 10^{-1} to 10^{-5} with virus dilutions 10^{-5} to 10^{-9}; these mixtures also were then incubated at 37° C. for 4 hours. The results of inoculation are shown in Table III, from which it will be seen that separate mixtures of immune serum dilutions 10^{-3}, 10^{-4}, 10^{-5} and virus dilutions 10^{-7}, 10^{-8} and 10^{-9} respectively failed to protect, while the same mixtures derived from undiluted immune serum and virus dilution 10^{-4} all protected.

Experiment IV.

This experiment was essentially a repetition of Experiment III in a somewhat more extended form. Undiluted immune serum was first mixed with dilutions of virus from 10^{-1} to 10^{-10}, while dilutions of immune serum 10^{-1},

10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were similarly mixed with virus dilutions 10^{-1} to 10^{-10}. These mixtures were each separately prepared, and after incubation at 37° C. inoculated intraperitoneally into batches of 4 mice. As a control, normal undiluted serum was mixed with virus dilutions 10^{-1} to 10^{-10}. From Table IV it will be seen that virus mixed with normal serum was active in a dilution of 10^{-9}, that undiluted immune serum inactivated all dilutions of virus up to and including virus 10^{-1}, but that immune serum 10^{-1} only completely inactivated virus 10^{-4}, virus 10^{-3} being partially active with this dilution of serum. Similarly, mixtures of virus 10^{-4} and immune serum 10^{-2} were active, while even mixtures of virus 10^{-9} and immune serum dilutions 10^{-3}, 10^{-4} and 10^{-5} were still partially active. As a further amplification of Experiment III undiluted immune serum was incubated for 4 hours at 37° C. with concentrations of virus 10^{-3}, 10^{-4} and 10^{-5}. These mixtures were then decimally diluted and inoculated into batches of 4 mice. It should be noted that dilution of the first member of the series therefore gives a series undiluted serum and virus 10^{-3}; serum 10^{-1} and virus 10^{-4}, etc.; the second member gives undiluted serum and virus 10^{-4}; serum 10^{-1} and virus 10^{-5}, etc.; and the third member undiluted serum and virus 10^{-5}, serum 10^{-1} and virus 10^{-6}, etc. The first member (undiluted serum and virus 10^{-3}) was, as shown in Table IV, partly dissociable on dilution, but the second and third (undiluted sera and virus 10^{-3} and 10^{-4}) were not dissociated on dilution. A series of mixtures (serum 10^{-3} and virus 10^{-7}, serum 10^{-3} and virus 10^{-8}; serum 10^{-4} and virus 10^{-8}, serum 10^{-4} and virus 10^{-9}; serum 10^{-5} and virus 10^{-8}, serum 10^{-5} and virus 10^{-9}) were now completely inactive, although previously in similar mixtures

TABLE II.

Serum and Virus Mixtures Diluted after 15 Minutes at Room Temperature.

Undil. S. V. 10^{-4}.	S. 10^{-1}. V. 10^{-5}.	S. 10^{-2}. V. 10^{-6}.	S. 10^{-3}. V. 10^{-7}.	S. 10^{-4}. V. 10^{-8}.
		Immune Serum and Virus.		
S	S	†3	†3	S
S	S	†3	†3	S
S	S	†4	S	S
S	S	S	S	S

Undil. S. V. 10^{-5}.	S. 10^{-1}. V. 10^{-6}.	S. 10^{-2}. V. 10^{-7}.	S. 10^{-3}. V. 10^{-8}.	S. 10^{-4}. V. 10^{-9}
		Immune Serum and Virus.		
S	S	S	S	S
S	S	S	S	S
S	S	S	S	S
S	S	S	S	S
		Normal Serum and Virus.		
†2	†2	†2	†3	†3
†2	†2	†2	†3	†3
†3	†3	†2	†3	†3
†3	†4	†3	†3	†4

TABLE II—*continued.*

Serum and Virus Mixtures Diluted after 4 Hours at 37° C.

Undil. S. V. 10^{-4}.	S. 10^{-1}. V. 10^{-5}.	S. 10^{-2}. V. 10^{-6}.	S. 10^{-3}. V. 10^{-7}.	S. 10^{-4}. V. 10^{-8}.
	Immune Serum and Virus.			
s	†5	†3	†3	s
s	s	†4	†3	s
s	s	†4	†3	s
s	s	s	s	s

Undil. S. V. 10^{-5}.	S. 10^{-1}. V. 10^{-6}.	S. 10^{-2}. V. 10^{-7}.	S. 10^{-3}. V. 10^{-8}.	S. 10^{-4}. V. 10^{-9}.
	Immune Serum and Virus.			
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s
	Normal Serum and Virus.			
†2	†2	†2	†2	†3
†2	†2	†2	†2	†3
†2	†2	†3	†3	†3
†3	†2	†3	†3	†3

S. = serum. V. = virus. † = death. s = survival.

TABLE III.

Undil. S. V. 10^{-4}.	S. 10^{-1}. V. 10^{-5}.	S. 10^{-2}. V. 10^{-6}.	S. 10^{-3}. V. 10^{-7}.	S. 10^{-4}. V. 10^{-8}.	S. 10^{-5}. V. 10^{-9}.
		Normal Serum and Virus.			
†2	†2	†3	†2	†3	†3
†2	†2	†3	†3	†3	†3
†3	†2	†3	†3	†4	s
†3	†3	†4	†3	s	s
		Immune Serum and Virus (separate dilution mixtures made before incubation).			
s	s	s	†3	†5	†6
s	s	s	†7	†5	†6
s	s	s	†8	†10	s
s	s	s	†8	s	s
		Immune Serum and Virus (dilution mixtures made after incubation from undiluted serum and virus 10^{-4} mixture).			
s	s	s	s	s	s
s	s	s	s	s	s
s	s	s	s	s	s
s	s	s	s	s	s

S. = serum. V. = virus. † = death. s = survival.

TABLE IV.

Dilutions of virus.	Normal serum and virus dilutions.	Immune serum and virus (mixtures made separately before incubation). Serum dilutions.						Immune serum and virus (mixtures derived after incubation from undiluted serum with virus diluted 10^{-4}, 10^{-4} and 10^{-5}). Serum dilutions.					
		Undil.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	Undil.	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
10^{-1}	++++	○○○○	++++	++++	++++	++++	++++	−	−	−	−	−	−
10^{-2}	++++	○○○○	++○○	+++○	++++	++++	++++	−	−	−	−	−	−
10^{-3}	++++	○○○○	+○○○	+++○	++++	++++	++++	○○○○	−	−	−	−	−
10^{-4}	++++	○○○○	○○○○	++++	++++	++++	++++	○○○○	++○○	−	−	−	−
10^{-5}	++++	○○○○	○○○○	○○○○	++++	++++	++++	○○○○	○○○○	++++	−	−	−
10^{-6}	++++	○○○○	○○○○	○○○○	++++	++++	++++	−	○○○○	○○○○	++++	−	−
10^{-7}	+++○	○○○○	○○○○	○○○○	++++	++++	++++	−	−	○○○○	○○○○	+++○	−
10^{-8}	++○○	○○○○	○○○○	○○○○	+++○	+++○	++++	−	−	−	○○○○	○○○○	○○○○
10^{-9}	++○○	○○○○	○○○○	○○○○	+○○○	++○○	++○○	−	−	−	−	○○○○	○○○○
10^{-10}	○○○○	○○○○	○○○○	○○○○	○○○○	○○○○	○○○○	−	−	−	−	−	○○○○

+ = death. ○ = survival. The entries underlined in the left-hand division of the table should be contrasted with those similarly underlined in the right-hand division. They show the difference in the results *before* and *after* incubation.

prepared before incubation virus was active. The conclusion is therefore reached that mixtures of virus and serum, prepared separately *before* incubation, are not necessarily inactive in concentrations where equivalent mixtures, derived *after* incubation by decimal dilution of non-dissociable mixtures of immune serum and virus, are necessarily inactive.

Experiment V.

From the previous experiments the conclusion may be drawn that reactivation by dilution is possible only when immune serum and virus are mixed together in certain proportions. When the amount of virus is decreased while the amount of immune serum remains constant, a point is reached at which reactivation by dilution no longer occurs. This point is reached in the present experiments when an amount of virus between 10^{-4} and 10^{-5} is mixed with an equal amount of undiluted immune serum. At this point there appears to be evidence of some union *in vitro* between the immune serum and all the virus particles.

There are, however, two curious phenomena which are unexplained by the foregoing experiments: (i) the apparent inactivity of a mixture of virus and serum inoculated intraperitoneally and the activity of the same mixture inoculated by another route—in the case of Rift Valley fever, intranasally; (ii) the apparent inactivity of a mixture of virus and serum *in vivo* when the same mixture can be reactivated *in vitro* by simple dilution.

In regard to the first phenomenon, the finding that reactivation by dilution is strictly quantitative suggested that reactivation by changing the route of injection might also be quantitative in character. A constant amount of immune serum was therefore mixed with falling dilutions of virus and the

TABLE V.

Normal Serum (undil.)

Intraperitoneal route.				Intranasal route.			
Virus Dilutions.							
10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
†3	†3	†2	†3	†3	†3	†4	†4
†3	†3	†3	†3	†4	†4	†4	†5
†3	†3	†3	†4	†5	†5	†5	†5
†3	†4	†4	†4	†5	†6	†5	†5

Immune Serum (undil.)

Intraperitoneal route.				Intranasal route.			
Virus Dilutions.							
10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
s	s	s	s	†4	†5	s	s
s	s	s	s	†4	†7	s	s
s	s	s	s	†5	s	s	s
s	s	s	s	†9	s	s	s

† = death. s = survival.

mixtures incubated at 37° C. for 4 hours. As a control, normal human serum was mixed with the same dilutions of virus and incubated. After incubation 0·4 c.c. of each mixture was inoculated intraperitoneally into each of a batch of 4 mice, while 0·03 c.c. of each similar mixture was instilled intranasally into each of another batch of 4 mice, the mice being under ether anæsthesia.

The results are shown in Table V. It will be seen that mixtures of virus and immune serum which are inactive when inoculated intraperitoneally may nevertheless be active when instilled intranasally, but only with virus dilutions greater than 10^{-4}. As in the case of reactivation by dilution, the phenomenon of reactivation by change of route of injection is also quantitative.

Experiment VI.

The next point to be determined was whether reactivation by dilution exactly parallels reactivation by change of route of injection.

With this object, amounts of undiluted immune serum were mixed with equal amounts of virus dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}; these mixtures were incubated at 37° C. for 4 hours and then diluted decimally, so that in each case the final virus dilution was 10^{-9}. These decimal dilutions were then inoculated intraperitoneally into mice in doses of 0·4 c.c. per mouse. In addition, the mixtures of undiluted immune serum and virus dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were instilled intranasally into mice in doses of 0·03 c.c. As controls, normal undiluted human serum was mixed with virus diluted 10^{-2}, incubated for 4 hours at 37° C. and then decimally diluted to a final concentration of virus 10^{-9}; undiluted normal human serum was also inoculated with equal amounts of virus dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}, and after the same period of incubation these mixtures were instilled intranasally into mice in doses of 0·03 c.c. Results are detailed in Table VI, from which it is seen that while a mixture of undiluted immune serum and virus 10^{-3} could be reactivated by dilution, this did not occur with the mixture of undiluted immune serum and virus 10^{-4}. At the same time the mixtures of undiluted serum and virus 10^{-2} and 10^{-3}, though inactive when injected intraperitoneally, were active when instilled intranasally, but the mixture of undiluted serum and virus 10^{-4} was inactive both on intraperitoneal injection and on intranasal instillation. Reactivation by dilution and by alteration in the route of inoculation thus both occur only in the same mixtures of immune serum and virus. It is therefore not improbable that the two phenomena are fundamentally similar.

TABLE VI.

Normal Serum and Virus.

Intraperitoneal inoculation 0·4 c.c.

Undil. S. V. 10^{-2}.	S. 10^{-1}. V. 10^{-3}.	S. 10^{-2}. V. 10^{-4}.	S. 10^{-3}. V. 10^{-5}.	S. 10^{-4}. V. 10^{-6}.	S. 10^{-5}. V. 10^{-7}.	S. 10^{-6}. V. 10^{-8}.	S. 10^{-7}. V. 10^{-9}.
†2	†3	†2	†2	†3	†3	†3	†3
†3	†4	†3	†3	†3	†3	†3	†3
†3	†4	†4	†3	†3	†3	†3	†4
†3	†6	†4	†5	†4	†3	†5	†6

TABLE VI—*continued.*

Intranasal inoculation 0·03 c.c.

Undil. S. V. 10^{-2}.	Undil. S. V. 10^{-3}.	Undil. S. V. 10^{-4}.	Undil. S. V. 10^{-5}.
†3	†3	†4	†4
†4	†3	†4	†4
†4	†4	†5	†4
†5	†5	†5	†5

Immune Serum and Virus.

Intraperitoneal inoculation 0·4 c.c.

Undil. S. V. 10^{-2}.	S. 10^{-1}. V. 10^{-3}.	S. 10^{-2}. V. 10^{-4}.	S. 10^{-3}. V. 10^{-5}.	S. 10^{-4}. V. 10^{-6}.	S. 10^{-5}. V. 10^{-7}.	S. 10^{-6}. V. 10^{-8}.	S. 10^{-7}. V. 10^{-9}.
s	†6	†5	†4	†3	†3	s	s
s	†6	†8	†4	†3	†3	s	s
s	s	s	†5	†3	s	s	s
s	s	s	†6	s	s	s	s

Undil. S. V. 10^{-3}.	S. 10^{-1}. V. 10^{-4}.	S. 10^{-2}. V. 10^{-5}.	S. 10^{-3}. V. 10^{-6}.	S. 10^{-4}. V. 10^{-7}.	S. 10^{-5}. V. 10^{-8}.	S. 10^{-6}. V. 10^{-9}.
s	†5	†6	†5	†5	s	s
s	s	s	†5	s	s	s
s	s	s	†6	s	s	s
s	s	s	†6	s	s	s

Undil. S. V. 10^{-4}.	S. 10^{-1}. V. 10^{-5}.	S. 10^{-2}. V. 10^{-6}.	S. 10^{-3}. V. 10^{-7}.	S. 10^{-4}. V. 10^{-8}.	S. 10^{-5}. V. 10^{-9}.
s	s	s	s	s	s
s	s	s	s	s	s
s	s	s	s	s	s
s	s	s	s	s	s

Undil. S. V. 10^{-5}.	S. 10^{-1}. V. 10^{-6}.	S. 10^{-2}. V. 10^{-7}.	S. 10^{-3}. V. 10^{-8}.	S. 10^{-4}. V. 10^{-9}.
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s

Intranasal inoculation 0·03 c.c.

Undil. S. V. 10^{-2}.	Undil. S. V. 10^{-3}.	Undil. S. V. 10^{-4}.	Undil. S. V. 10^{-5}.
†4	†5	s	s
†4	†9	s	s
†5	†9	s	s
†9	†9	s	s

V. = virus. S. = serum. † = death. s = survival.

Experiment VII.

In studying the results obtained in the two previous experiments, it is noticeable that in addition to the difference in the routes of administration, there is also involved a considerable difference in the doses of the serum-virus mixtures inoculated, 0·4 c.c. and 0·03 c.c. respectively. It therefore appeared possible that this variation might be of greater significance than the actual difference in the routes of administration. An experiment was therefore carried out to see whether the intraperitoneal inoculation of virus-serum mixtures in doses of 0·4 c.c. and 0·03 c.c. would lead to any difference in regard to the pathogenicity of the mixtures. With this object, mixtures of serum and virus were made up as in Experiment V, and 0·4 c.c. of each mixture was inoculated intraperitoneally into 4 mice ; at the same time 4 other mice were given an interperitoneal injection of 0·03 c.c. of the same mixture. The results are shown in Table VII.

TABLE VII.

Normal Serum (undil.) and Virus.

Intraperitoneal inoculation 0·4 c.c.

Virus Dilutions.				
10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
†2	†2	†2	†3	†3
†3	†2	†2	†3	†3
†3	†3	†3	†3	†3
†3	†3	†3	†3	†3

Intraperitoneal inoculation 0·03 c.c.

†2	†2	†3	†3	†3
†3	†3	†3	†3	†3
†3	†3	†3	†3	†3
†3	†3	†3	†4	s

Immune Serum (undil.) and Virus.

Intraperitoneal inoculation 0·4 c.c.

Virus Dilutions.				
10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s

Intraperitoneal inoculation 0·03 c.c.

†11	†5	†5	s	s
s	†10	†9	s	s
s	s	†11	s	s
s	s	s	s	s

† = death. s = survival.

It will be seen that with normal serum and virus all the mixtures were pathogenic whether given in doses of 0·4 c.c. or 0·03 c.c. When, instead of normal serum, immune serum and virus mixtures were injected, the larger injections completely protected the mice, but after a prolonged incubation period there was mortality among the mice inoculated with virus diluted 10^{-1}, 10^{-2} and 10^{-3}. A small dose of virus and immune serum was more likely to be pathogenic than a large dose of the same mixture. This suggests that the infectivity of apparently neutral mixtures when instilled intranasally is due to the small amount of virus-serum mixture absorbed in a given time and not so much to the actual route of inoculation.

Since in Table VI it was found that intranasal infectivity was exhibited only by those mixtures of immune serum and virus that could be dissociated or reactivated by dilution *in vitro*, it seemed to be of importance to determine whether reactivation of small (0·03 c.c.) injections also occurred only with mixtures dissociable by dilution *in vitro*.

Experiment VIII.

In this experiment the previous experiment was repeated, except that after incubation the mixture of normal human serum and virus dilution 10^{-2} and the mixtures of immune human serum and virus dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} were decimally diluted with saline before inoculation. The results are given in Table VIII, from which it is seen that only those mixtures of immune serum and virus dilutions 10^{-2} and 10^{-3} which are reactivated on dilution *in vitro* are also reactivated when injected intraperitoneally in doses of 0·03 c.c. The results are thus directly comparable with those recorded in Table VI.

Experiment IX.

From the results obtained in the previous experiments it obviously became necessary to attempt an explanation of why a mixture of immune serum and virus which can be reactivated by dilution *in vitro* is only dissociated *in vivo* when the inoculum is small but not when it is large. One possible explanation which suggested itself was the great difference in the amount of virus capable of acting as antigen in the two inoculums, Mackenzie (1934) having shown that in order to obtain immunity in mice with killed Rift Valley fever virus it was essential to employ a very large amount of antigen, which was proportional to the body-weight.

During the course of the investigations here recorded a number of mice had been obtained that had survived varying doses of immune serum and virus. As a matter of interest, in from 14 to 21 days after the injection of immune serum and virus, when all passive immune body would have been excreted, it had been customary to find out whether the survivors showed any immunity by giving them an intraperitoneal inoculation of 0·2 c.c. of a 10^{-2} suspension of infected mouse liver in saline. As a similar injection of virus diluted 10^{-9} or 10^{-10} represented the end-point, this test inoculum represented at least 10,000,000 M.L.D. The mice were observed for 14 days after the virus inoculation, and those that survived this period were

Table VIII.

Normal Serum and Virus Intraperitoneal Inoculation. 0·4 c.c.

Undil. S. V. 10^{-2}.	S. 10^{-1}. V. 10^{-3}.	S. 10^{-2}. V. 10^{-4}.	S. 10^{-3}. V. 10^{-5}.	S. 10^{-4}. V. 10^{-6}.	S. 10^{-5}. V. 10^{-7}.	S. 10^{-6}. V. 10^{-8}.	S. 10^{-7}. V. 10^{-9}.
†2	†2	†2	†3	†3	†3	†3	†3
†2	†3	†3	†3	†3	†3	†3	†3
†3	†4	†3	†3	†3	†3	†4	†5
†3	†4	†4	†4	†3	†4	†4	†6

0·03 c.c.

Undil. S. V. 10^{-2}.	Undil. S. V. 10^{-3}.	Undil. S. V. 10^{-4}.	Undil. S. V. 10^{-5}.
†2	†2	†2	†3
†2	†3	†3	†3
†3	†3	†3	†4
†3	†3	†4	†4

Immune Serum and Virus Intraperitoneal Inoculation. 0·4 c.c.

Undil. S. V. 10^{-2}.	S. 10^{-1}. V. 10^{-3}.	S. 10^{-2}. V. 10^{-4}.	S. 10^{-3}. V. 10^{-5}.	S. 10^{-4}. V. 10^{-6}.	S. 10^{-5}. V. 10^{-7}.	S. 10^{-6}. V. 10^{-8}.	S. 10^{-7}. V. 10^{-9}.
s	†5	†5	†4	†4	†3	†6	s
s	s	†6	†5	†4	†4	s	s
s	s	†8	†5	†5	s	s	s
s	s	s	†6	†5	s	s	s

Undil. S. V. 10^{-3}.	S. 10^{-1}. V. 10^{-4}.	S. 10^{-2}. V. 10^{-5}.	S. 10^{-3}. V. 10^{-6}.	S. 10^{-4}. V. 10^{-7}.	S. 10^{-5}. V. 10^{-8}.	S. 10^{-6}. V. 10^{-9}.
s	†6	†5	†4	†5	†8	s
s	†6	†6	†6	†6	s	s
s	s	†6	†7	s	s	s
s	s	s	†7	s	s	s

Undil. S. V. 10^{-4}.	S. 10^{-1}. V. 10^{-5}.	S. 10^{-2}. V. 10^{-6}.	S. 10^{-3}. V. 10^{-7}.	S. 10^{-4}. V. 10^{-8}.	S. 10^{-5}. V. 10^{-9}.
s	s	s	s	s	s
s	s	s	s	s	s
s	s	s	s	s	s
s	s	s	s	s	s

Undil. S. V. 10^{-5}.	S. 10^{-1}. V. 10^{-6}.	S. 10^{-2}. V. 10^{-7}.	S. 10^{-3}. V. 10^{-8}.	S. 10^{-4}. V. 10^{-9}.
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s
s	s	s	s	s

0·03 c.c.

Undil. S. V. 10^{-2}.	Undil. S. V. 10^{-3}.	Undil. S. V. 10^{-4}.	Undil. S. V. 10^{-5}.
†5	†6	s	s
†9	†10	s	s
†10	†10	s	s
†11	s	s	s

S. = serum. V. = virus. † = death. s = survival.

regarded as showing some degree of immunity. The results shown in Table IX show that the chances of survival are proportional to the original dose of virus used in the virus-serum mixture.

TABLE IX.—*The Action of a Test Inoculation of Virus on Mice Previously Injected with Varying Concentrations of Virus and Immune Serum.*

Concentration of virus in immunizing inoculum.	Result of test inoculation of virus.	
	Survival.	Death.
10^{-2}	27	3
10^{-3}	20	10
10^{-4}	13	17
10^{-5}	1	29
10^{-6}	0	30
10^{-7}	0	30
10^{-8}	0	30
10^{-9}	0	30

DISCUSSION.

The two phenomena which caused these experiments to be undertaken, the possibilities, namely, of reactivating an apparently neutral mixture of Rift Valley fever virus and immune serum either by dilution *in vitro* or by changing from an intraperitoneal to an intranasal route of inoculation, have been found to occur, but only under certain circumstances. When to constant amounts of immune serum there are added decreasing amounts of virus, a point is reached at which the mixture can no longer be reactivated either by dilution *in vitro* or by varying the route of injection. In the present series of experiments, this end-point was reached when undiluted serum was mixed with an equal quantity of virus diluted either $0{\cdot}5 \times 10^{-4}$ or $0{\cdot}5 \times 10^{-5}$, although in control experiments with normal serum, virus was fully active in these concentrations. It is therefore assumed that in these circumstances some union occurs *in vitro* between the immune serum and the virus, and that this union cannot be dissociated by simple dilution. In the course of this investigation, it was also found that certain mixtures of virus and immune serum which are non-pathogenic when injected intraperitoneally in large doses are nevertheless active when injected by the same route in small doses. This phenomenon also only occurred with those virus-serum mixtures that can be reactivated by dilution or by intranasal inoculation. Since by this latter route only a small amount of virus-serum mixture can be instilled, it seems probable, in view of the results obtained with different amounts by the intraperitoneal route, that the amount of the inoculum is of greater importance than the route. It remains to explain why a small injection of virus and serum should be more liable to produce pathogenic results than a large injection of the same mixture. Theiler and Whitman (1935) have already noticed this apparent contradiction in relation to the neurotropic yellow fever virus and its specific immune serum.

The following hypothesis is suggested to explain these facts: When a

large inoculum of immune serum and virus is injected, a certain number of cells are immediately invaded by the virus, which proceeds to grow in these cells. Virus will be liberated from these cells, and at the same time immune bodies will be produced from the tissues. Virus so liberated meets with the immune bodies present in the serum and, provided the proportion of immune bodies and virus is correct, there forms a non-dissociable mixture. The immune bodies are provided both by the foreign serum originally injected and the immune bodies formed from the animal's own tissues. As the passively injected immune bodies are excreted, the actively produced immune bodies take their place, and thus a balance is struck between the amount of virus and the amount of immune bodies. Death does not take place.

When a small quantity of virus and immune body is inoculated, the same process occurs as before, but now the small amount of passively injected immune bodies disappears more rapidly from the circulation, while the small amount of virus produces in the same time a comparatively small amount of immune body. A point is thus reached when there is no longer any passive immune body left and the amount of active immune body is very small. Virus is therefore present in an active condition, and death can occur.

This hypothesis is at present being tested by experiments which will form the subject of a further communication.

Evidence derived from the present experiments does, however, show that when an animal receives a mixture of virus and immune serum its fate depends not only on the relative concentration of virus and immune serum in the mixture, but on the actual amount of the inoculum when that inoculum contains a virus-serum mixture that can be reactivated by dilution.

SUMMARY.

1. As the result of acquired active immunity, Rift Valley fever immune bodies can be demonstrated in the serum of human beings 4 to 5 years after infection.

2. Certain mixtures of Rift Valley fever virus and immune serum, apparently neutral when injected intraperitoneally, can be reactivated or dissociated by simple dilution with saline.

3. This phenomenon is quantitative; for if falling dilutions of virus are added to equal amounts of undiluted immune serum, a point is reached at which the mixture can no longer be reactivated or dissociated by simple dilution.

4. Mixtures, separately prepared before incubation at 37° C., and containing dilutions of virus and immune serum equivalent to those derived, after incubation, by decimal dilution of a non-dissociable mixture of undiluted immune serum and virus, were active when inoculated intraperitoneally.

5. Certain mixtures of Rift Valley fever virus and immune serum, apparently neutral when injected intraperitoneally, can be reactivated by intranasal instillation.

6. This phenomenon is also quantitative, and can only be demonstrated with those serum-virus mixtures that can be reactivated by simple dilution.

7. Certain mixtures of Rift Valley fever virus and immune serum, apparently

neutral when injected intraperitoneally in doses of 0·4 c.c., are found to be active when injected intraperitoneally in doses of 0·03 c.c.

8. This phenomenon is also quantitative, and can only be demonstrated with those serum-virus mixtures that can be reactivated by simple dilution.

9. The reactivation of an apparently neutral mixture by intranasal instillation is probably due not so much to the route of inoculation as to the small quantity of the inoculum.

10. Evidence is brought forward to show that mice that have survived an injection of virus and immune serum are immunized only when the amount of virus in the mixture injected exceeds a certain minimum.

11. The above findings are briefly discussed, and an hypothesis is put forward to explain the divergent results following the intraperitoneal injection of varying serum-virus mixtures.

My thanks are due to A. E. Green and I. A. Hepple for a generous supply of Rift Valley fever immune serum.

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PARATHORMONE TOLERANCE IN DOGS.

N. B. TAYLOR, C. B. WELD AND J. F. SYKES.
From the Department of Physiology, University of Toronto.

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ADMINISTRATION OF IRRADIATED ERGOSTEROL TO PARATHORMONE-TOLERANT ANIMALS.

IF the toxic action of large doses of irradiated ergosterol be due to any appreciable extent to stimulation of the parathyroid glands, then it would seem reasonable to suppose that animals rendered tolerant to parathormone would also be resistant to irradiated ergosterol.

To test this hypothesis we administered gradually increasing doses of parathormone to three dogs, starting with 0·1 c.c. (2 units) per day, and increasing the quantity at weekly intervals provided that no, or at the most only a

transient, hypercalcæmia resulted from the dosage of the previous week. At the end of two months dog 225 was receiving 2 c.c. daily, and after five months dog 250 was being given 3 c.c. daily ; both of these animals appeared normal, and had good appetites and normal serum calcium. Dog 227, on the other hand, was with such difficulty brought to the 1-c.c. level that no larger doses of parathormone were given to this animal. Even this dose caused a lessened appetite and a serum calcium tending to be slightly elevated. It is interesting to note that in the case of dog 250 some information may be gleaned as to the duration of the tolerance. When the 2-c.c. level of dosage was established, parathormone administration was discontinued for six weeks. At the end of that time the animal was still tolerant of 1 c.c. daily, which was then given, and it was found possible to increase the dose rapidly to 3 c.c. daily without ill-effect.

When three animals had been obtained which were tolerant of doses of parathormone that would usually prove fatal, massive doses of irradiated ergosterol were given and the animals further observed. The amounts used (0·4 c.c. 10,000 X, or 400,000 I.U. per kg., for three doses) have in our experience proved invariably fatal when given to normal animals. All three animals survived. The process was repeated a second time in the case of dog 225 after a further course of parathormone, but proved fatal on this occasion. These results are shown in Table I, and to us it seems very significant that the only dogs we have known to survive such doses of irradiated ergosterol are these three which were tolerant to considerable doses of parathormone.

TABLE I.—*Ergosterol Overdosage to Parathormone-tolerant Dogs.*

Dog.	Dose.	Result.
225 (tolerant to 2 c.c. parathormone)	0·4 c.c. 10,000 X per kg. 3 doses alternate days	Survived.
227 (tolerant to 1 c.c. parathormone)	0·4 c.c. 10,000 X per kg. 3 doses alternate days	,,
225 (tolerant to 3 c.c. parathormone)	0·4 c.c. 10,000 X per kg. 3 doses consecutive days	Died in 6 days.
250 (tolerant to 3 c.c. parathormone)	0·4 c.c. 10,000 X per kg. 3 doses consecutive days	Survived.

The converse of this experiment is now in progress. Considerable difficulty has been experienced in obtaining dogs tolerant to large doses of ergosterol, and to date only one has shown any tolerance. This one was finally receiving 0·12 c.c. 10,000 X per kg. daily without loss of appetite when we gave killing doses of parathormone. He survived for six days, receiving 3 c.c. daily, which is longer than the survival time of any other dog in our records for such a dose, and this despite the fact that he weighed but 6 kg., having lost 40 p.c. of his body-weight during the five months of ergosterol administration.

POSSIBILITY OF PASSIVE TRANSFER OF PARATHORMONE TOLERANCE.

From time to time serum was obtained from these parathormone-tolerant animals, pooled and preserved in the refrigerator. The pooled serum was then used in an attempt to ascertain whether it contained any " antiparathyroid activity ".

(*a*) It was injected alone to show if it would depress the serum calcium, both into normal dogs and into one presumably rendered sensitive to a calcium-lowering action by unilateral extirpation of the thyroparathyroid glands. The details are shown in Table II, from which it may be seen that in the six trials there was a fall in the serum calcium on two occasions, and a rise in one instance.

TABLE II.—*Serum Calcium Changes Following Injection of Serum from Parathormone-tolerant Dogs.*

Animal.	Amount of serum.	Serum Ca change.
254 (normal)	40	−1·1 (17 hours)
254 ,,	50	−0·4 (18 ,,)
254 ,,	30	−0·4 (18 ,,)
274 ,,	65	+0·2 (18 ,,)
255 (partially parathyroidectomized)	50	−2·0 (24 ,,)
255 ,,	50	+1·2 (24 ,,)

(*b*) The pooled serum was injected into normal dogs in conjunction with parathormone, to show whether it would modify the serum-calcium rise or the duration of survival. Dog 252 received 2 c.c. parathormone with 10 c.c. serum on two consecutive days and died in four. Dog 254 received 1 c.c. parathormone with 20 c.c. serum on three consecutive days and died in four. Both these animals exhibited a normal parathormone response as measured by duration of life, and also by the serum calcium curves, as seen from Fig. 1. Dog 273 was given a single 5-c.c. dose of parathormone, and his serum calcium curve followed, and then 10 days later this was repeated, but with 100 c.c. serum given 3 hours before the parathormone. As is seen from Fig. 2, there was not the slightest modification of the serum calcium curve, despite the serum administration.

(*c*) The pooled serum was injected in conjunction with a continuous intravenous infusion of calcium lactate solution. From Table III it is seen that the rate of rise of the serum calcium during continuous infusion of a calcium solution at a constant rate is definitely less in dogs tolerant to parathormone than in normal animals. Of the animals only 227 was hypercalcæmic at the time of the infusion. The solution injected into dogs 129, 227 and 272 contained 300 mg. Ca per 100 c.c., while the concentration of that given to 126, 250 and 274 was twice as great. In other words, a parathormone-tolerant dog differs from a normal dog in its serum calcium response to an intravenous calcium injection. This difference is not shown by normal dogs who have

been given serum from tolerant animals. Table IV shows this result. The normal serum calcium response to continuous intravenous calcium infusion

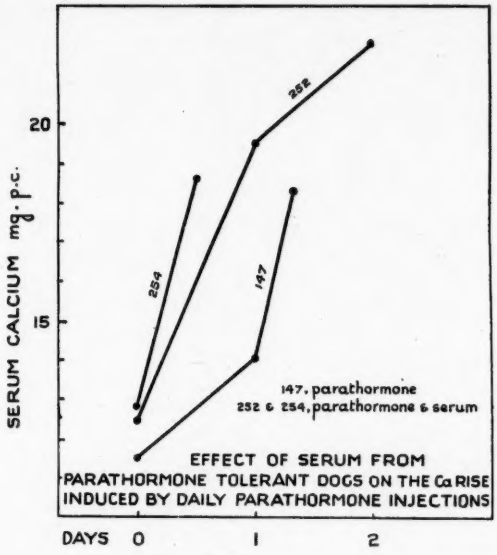

FIG. 1.

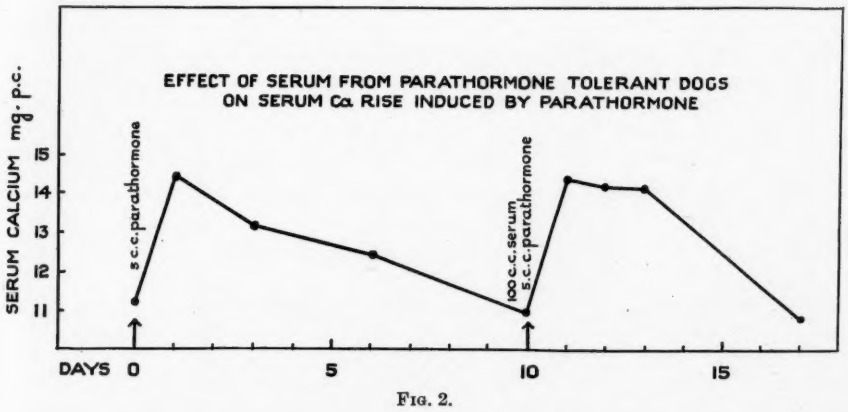

FIG. 2.

was determined on two dogs, and a week later this was repeated, the dogs on this occasion having been given 65 c.c. serum. There is no difference between the responses obtained before and after serum injection.

TABLE III.—*Ca Infusion to Parathormone-tolerant Dogs.*

Animal.	Rate of infusion	Serum calcium change. 2 hours.	4 hours.	6 hours.
129 (control)	30 mg. per hour	0	+5·0	..
227 (tolerant)	,, ,,	0	−0·5	−1·6
126 (control)	120 mg. per hour	+9·5	..	..
250 (tolerant)	,, ,,	+1·4	+3·4	+5·0
250 ,,	,, ,,	+2·5	+3·0	+3·2
250 ,,	225 mg. per hour	+4·2	..	..

TABLE IV.—*Ca Infusion to Dogs Before and After Injections of Serum from Parathormone-tolerant Animals.*

Animal.	Serum Ca change.
274 Ca infusion	+4·5
274 ,, + serum	+4·0
272 Ca infusion	+1·5
272 ,, + serum	+2·6

HYPERCALCÆMIA.

In the process of rendering dogs tolerant to parathormone the serum calcium level was found to be a suitable indication of safety if, at the most, only transient rises occurred when the dose was increased. Too great a rise or a prolonged rise always indicated the need of a reduction in dosage. These animals, however, when receiving large doses of irradiated ergosterol gave the usual hypercalcæmic response, the serum calcium remaining at high level for weeks. Dog 227, in fact, maintained a serum calcium level of 18 mg. per 100 c.c. or more for 44 days, and then under the influence of a series of parathormone injections maintained an equally high level for a further 41 days. None of these animals showed subjective signs of irradiated ergosterol or parathormone overdosage during this prolonged period of hypercalcæmia.

TABLE V.—*Duration of High Serum Calcium.*

	Parathormone. Type of dose.	Duration of high Ca.	Ergosterol. Type of dose.	Duration of Ca > 18 mg. %.
250	Tolerance	Transient	Excess (3 doses)	18 days
225	,,	,,	,, ,,	29 ,,
227	,,	*1 >20 mg. %=14 days; >15 mg. %=32 days	,, ,,	44 ,,
	,,	2 >18 mg. %=41 days		
246	..	..	Tolerance	35 ,,
271	..	..	,,	48 ,,
270	..	..	,,	117 ,,

* 1 and 2 = separate periods of treatment; total duration of period 1 = 32 days.

In the attempt to produce animals tolerant to irradiated ergosterol a hypercalcæmia soon developed, which persisted in spite of a reduction in dosage. These animals appeared well and normally active and showed no symptoms of overdosage except a transient loss of appetite, despite the hypercalcæmia of 18 mg. per 100 c.c. or more lasting for weeks. The serum calcium level was abandoned as an indication of the ability of the animal to withstand a given dose of irradiated ergosterol, more reliance being placed upon the general appearance and the appetite of the animal. These results are shown in Table V.

SUMMARY.

Tolerance to large daily doses of parathormone has been obtained in three dogs. These animals have been shown to survive massive doses of irradiated ergosterol.

No evidence has been obtained indicating that this tolerance can be passively transferred to normal animals. Serum from the tolerant animals when injected into normal dogs—

(*a*) failed to lower the serum calcium ;

(*b*) failed to prolong life or to modify the hypercalcæmia following parathormone injections ;

(*c*) failed to modify the serum calcium response to continuous intravenous infusion of calcium lactate solution.

Hypercalcæmia in itself is compatible with life, and is not responsible for the toxic effects of irradiated ergosterol.

OBSERVATIONS BEARING ON THE ANTIGENIC COMPOSITION OF PSITTACOSIS VIRUS.

S. P. BEDSON.

From the Bacteriological Department, London Hospital and Medical College.

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During the investigation of the immunity reactions of psittacosis virus some facts have come to light which suggest that this virus possesses more than one antigen. This, perhaps, was not unexpected ; already there was evidence that vaccinia virus is antigenically complex (Smith, 1932 ; Craigie and Wishart 1934*a* ; Hughes, Parker and Rivers, 1935). The antigenic analysis of psittacosis virus is, as yet, incomplete ; no attempt has been made to ascertain its chemical composition. It is thought, however, that the results so far obtained are of sufficient interest to be reported in their present state, leaving the more detailed analysis for a future investigation.

EXPERIMENTAL.

Complement Fixation.

It has been shown that a crude suspension of psittacosis virus, such as a suspension of virulent mouse spleen, will give a specific complement-fixation reaction with an anti-psittacosis serum (Bedson, 1933), and that this reaction can be made use of in the diagnosis of human infections with this virus (Bedson, 1935). An attempt has been made to find out what material in these crude suspensions is responsible for this reaction. It is known that the virus in such a suspension, when freed from the bulk of extraneous matter by fractional centrifugation and washing, will fix complement specifically (Bedson, 1932). But the reactions obtained with such an antigen have not been as good as those given by the original suspension, suggesting that there is some antigenic material other than elementary bodies in the crude suspension, which takes part in the fixation of complement. Crude virus suspension has been submitted to centrifugation, filtration and heating at different temperatures, and the resulting products tested for virulence and ability to fix complement.

Centrifugation.—Crude suspensions of virulent mouse spleen were centrifuged at various speeds and the supernatant fluid pipetted off, the deposit resuspended to the original volume and the fractions so obtained tested for virulence and complement-fixing power. The technique of the complement-fixation test was that previously described (Bedson and Bland, 1929). The psittacosis antiserum was prepared in the guinea-pig by intraperitoneal injection of a suspension of virulent guinea-pig spleen. From 10 to 15 inoculations were required to produce a serum of reasonable potency. The dose of complement throughout was 2 M.H.D. Tests for virulence were made by inoculating 0·2 c.c. quantities of decimal dilutions in the shaved skin of a guinea-pig's flank.

The results of two quite typical experiments are given in Table I.

It seems clear from these experiments that the crude suspensions contain material, other than formed active virus, which gives specific complement fixation. This material is less susceptible to centrifugal force than the elementary bodies but cannot be in a very high degree of dispersion, since it is slowly deposited by a speed of 10,000 r.p.m.

Seitz filtration.—The effect of filtration through a Seitz EK disc on virulence and complement-fixing power was next observed. Crude virus suspensions which had been clarified by a short centrifugation were passed through Seitz filters. The filters were either untreated, or submitted to a preliminary treatment with rabbit serum. This consisted of filtering 20 c.c. of 1 in 10 rabbit serum in saline and washing the filter with two lots of 20 c.c. saline. Whether the filter disc had been treated or not the results were the same ; both virulence and complement-fixing power were removed by Seitz filtration. An experiment which is typical of a number is given in Table II.

It seems clear, then, that the antigenic material present in a crude suspension of psittacosis virus and capable of giving a complement-fixation reaction consists of formed active virus—elementary bodies—and smaller particles which are devoid of virulence, are less readily thrown down by centrifugation,

TABLE I.

Antigens.		Fixation of complement. Dilutions of anti-psittacosis serum. 1/4.	1/8.	1/16.	1/32.	1/64.	Virulence. Highest dilution giving a reaction in guinea-pig's skin.
Crude suspension P. XV 914/5 . .		++++	++++	++++	++++	++++	1/1000
		++++	++++	++++	++++	+++	
P. XV 914/5 centrifuged for 1 hour at 5000 r.p.m.	Supernatant	++++	++++	++++	++++	+++	1/1
		++++	++++	+++	+++	++	
	Deposit .	+++	+	±	−	−	1/100
		++	±	∓	−	−	
Crude suspension P. XV 942/3 . .		++++	++++	++++	++++	++	1/1000
		++++	++++	+++	++	+	
P. XV 942/3 centrifuged for 1 hour at 10,000 r.p.m.	Supernatant	++++	++++	++	±	−	<1/1
		+++	++	±	−	−	
	Deposit .	++++	++++	++	+	−	1/1000
		+++	++	+	−	−	

++++ to ± indicate the degree of fixation. Readings after 30 minutes at 37° C. (upper row), and after standing overnight at room temperature (lower row).

TABLE II.

Antigens.		Fixation of complement. Dilutions of anti-psittacosis serum. 1/4.	1/8.	1/16.	1/32.	1/64.	Virulence. Highest dilution giving a reaction in guinea-pig's skin.
P. XV 954/5	Crude . . .	++++	++++	++++	++++	++++	1/1000
		++++	++++	++++	+++	++	
	Seitz filtrate . .	−	−	−	−	−	<1/1
		−	−	−	−	−	

but are removed by Seitz filtration. These latter particles probably result from virus breakdown. Craigie (1932) has shown that a Seitz filtrate of a suspension of vaccinia virus contains material of virus origin which gives a specific precipitin reaction with antivaccinia serum. Further, Craigie and Wishart (1934*b*) have found that such filtrates fix complement equally well. It is true that in the experiments of Craigie and Wishart fixation was allowed to take place for sixteen hours at 4° C., whereas in the experiments with psittacosis filtrates fixation was made at room temperature for three hours. This difference in technique was not responsible for the divergent results, since experiments with psittacosis filtrates carried out at 4° C. overnight gave completely negative results. It is perhaps significant that Craigie and Wishart were using an antiserum prepared in the rabbit, whereas the psittacosis serum was of guinea-pig origin. Pneumococcal antisera made in the rabbit differ from those made in the horse (Horsfall and Goodner, 1935). Whereas both give a precipitin reaction with the specific polysaccharide, only the serum made in the rabbit will fix complement with such an antigen. This peculiar behaviour of rabbit-made pneumococcal antiserum remains unexplained, but possibly antiviral sera made in the rabbit behave in a similar manner, and this might account for the different findings with vaccinia and psittacosis filtrates. The explanation of the negative results with the latter filtrates is not that specific antigen is absent, as will be seen from experiments to be described later. It seemed possible that the high degree of dispersion of the psittacosis antigen in the filtrates might be responsible for the negative complement-fixation results. If one could adsorb it on to larger particles it might behave differently. Previous work had shown that it was possible to sensitize heat-killed colon bacilli by treating them with a crude virus suspension. Bacilli so treated gave specific complement fixation even after they had been washed several times. The same type of experiment was made with Seitz filtrates. Heat-killed (60° C.) colon bacilli were suspended in a Seitz filtrate of a crude suspension of psittacosis virus and the mixture placed in the refrigerator (4° C.) overnight. The bacilli were then centrifuged out, washed three times with saline and resuspended in saline to the original volume. A control was put up using the crude suspension in place of the filtrate in order to make it certain that under the conditions of the experiment the colon bacilli could adsorb psittacosis antigen. The various antigens—crude suspension, Seitz filtrate and the colon bacilli which had been in contact with them—were then tested for their power to fix complement in the presence of a psittacosis antiserum. Table III records the findings typical of a number of experiments.

Although the treatment of colon bacilli with the crude virus suspension renders them capable of reacting specifically in the presence of a psittacosis serum, similar treatment with a Seitz filtrate does not have this effect.

Heat.—The effect of different temperatures on the virulence and complement-fixing power of crude suspensions of virus was next determined. An example of the findings obtained is given in Table IV.

It is clear that virulence is destroyed at a lower temperature than that which interferes with the complement-fixing power of a crude virus suspension. The latter only disappears when the temperature has been sufficient to coagulate the proteins. When these suspensions of virulent mouse spleen are

TABLE III.

Sera. Nature.	Dilution.	Antigens. Crude suspension.	Seitz filtrate.	Untreated colon bacilli.	Colon bacilli treated with crude suspension.	Colon bacilli treated with Seitz filtrate.	Saline.
Anti-psittacosis	1/4	++++	∓	−	++++	−	−
		++++	−	−	++++	−	−
	1/8	++++	−	−	++++	−	
		++++	−	−	+++	−	
	1/16	++++	−	−	++	−	
		+++	−	−	+	−	
	1/32	+++	−	−	+	−	
		++	−	−	±	−	
Normal guinea-pig	1/4	∓	−	∓	∓	∓	∓
		−	−	−	−	−	−
Saline		−	−	−	−	−	−
		−	−	−	−	−	−

TABLE IV.

Treatment of crude virus suspensions.	Fixation of complement. Dilution of anti-psittacosis serum. 1/4.	1/8.	1/16.	1/32.	1/64.	Virulence. Titre in guinea-pig's skin.
Unheated	++++	++++	++++	++++	++	1/10,000
	++++	++++	++++	+++	+	
30 min. at 60° C.	++++	++++	++++	+++	+	<1/1
	++++	++++	++++	+++	±	
30 ,, 70° C.	++++	++++	++++	++++	++	<1/1
	++++	++++	++++	++++	+	
30 ,, 80° C.	−	−	−	−	−	<1/1
	−	−	−	−	−	
20 ,, 90° C.	−	−	−	−	−	<1/1
	−	−	−	−	−	
20 ,, 100° C.	−	−	−	−	−	<1/1
	−	−	−	−	−	

heated above 70° C. a considerable coagulum is formed, which rapidly settles out, leaving a clear supernatant fluid. Since it was this clear supernatant fluid which was used as antigen in the type of experiment given in Table IV, it does not necessarily follow from these results that temperatures above 70° C. destroy the complement-fixing power of the psittacosis antigen. The antigen might merely be carried down with the coagulated protein. An experiment was made to see if this were so. A portion of a crude virus suspension was adjusted to pH 6·0 with acetic acid and boiled for three minutes. The coagulum formed was thrown down by light centrifugation, the clear supernatant fluid pipetted off and its reaction adjusted to pH 7·6, and the deposit washed twice with saline. Smears made from the washed deposit and stained by Castañeda showed an abundance of elementary bodies, the majority of which were in loose clumps of varying size. The deposit was ground up and resuspended to the original volume in broth. Broth was used because it was with this diluent that the crude suspension had been made. The deposit gave a muddy suspension, which was rather unstable, and a considerable portion of the suspended matter soon settled out. This supension, in spite of its turbidity, was not anti-complementary, and when tested for specific complement-fixing power gave the result shown in Table V.

It is obvious from this experiment that the psittacosis antigen is carried down in the process of coagulating by heat the extraneous proteins in a crude virus suspension. It is equally clear that boiling for three minutes does not destroy its complement-fixing power. This experiment has been repeated several times with a similar result ; even prolonged boiling—up to one hour—has not destroyed this specific activity of the deposit. In these experiments the psittacosis antigen was in a crude state, mixed, as it was, with mouse spleen material. It was decided, therefore, to repeat them with a suspension of washed elementary bodies in place of the crude suspension. The elementary body suspension was obtained by fractional centrifugation and washing in the usual manner, an angle centrifuge running at 3000 r.p.m being employed for the purpose. The virus was washed twice and finally re-suspended to the original volume in broth. Castañeda preparations of the final suspension showed it to consist of discrete elementary bodies with little extraneous material. Portions of this suspension were heated at 60° C., 80° C. and 100° C., and the virulence and complement-fixing power compared with that of the unheated suspension. Table VI gives the result of such an experiment.

It is seen that the extremely poor fixing power of the washed elementary bodies is improved by heating and still further enhanced by boiling. This experiment has been repeated several times, and though the complement-fixing activity of the unheated elementary body suspension has varied, being sometimes quite good and sometimes as poor as, or even worse than, the result cited in Table VI, the boiled suspensions have invariably given good complement fixation. In this respect they have equalled or even surpassed the crude suspensions from which the elementary bodies were obtained. This improvement of the complement-fixing power of the elementary bodies brought about by boiling is difficult to explain. No naked-eye change appeared in the suspensions as the result of boiling, but smear preparations stained by Castañeda showed that the virus in the boiled suspensions was no longer evenly

TABLE V.

Antigens.	Fixation of complement. Anti-psittacosis serum. 1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	Normal guinea-pig serum. 1/4.	Saline.
Crude suspension	++++	++++	++++	++++	++++	++++	−	−
	++++	++++	++++	++++	++++	+++	−	−
Boiled for 3 min. — Supernatant	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−
Boiled for 3 min. — Deposit	++++	++++	++++	++++	++++	++	−	−
	++++	++++	++++	++++	++++	+	−	−
Saline	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−

TABLE VI.

Antigens.	Fixation of complement. Anti-psittacosis serum. 1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	Normal guinea-pig serum. 1/4.	Saline.	Virulence for guinea-pig skin.
Crude suspension	++++	+++	++++	++++	++++	++++	−	−	++
	++++	++++	++++	++++	++++	+++	−	−	
Washed elementary bodies — Unheated	+++	+	−	−	−	−	−	−	++
	++	±	−	−	−	−	−	−	
Heated for 30 min. at 60° C.	+++	++	+	±	∓	−	−	−	−
	++	+	±	−	−	−	−	−	
Heated for 20 min. at 80° C.	++++	++++	+++	++	+	±	−	−	−
	+++	+++	++	+	±	∓	−	−	
Heated for 20 min. at 100° C.	++++	++++	++++	++++	++++	+++	−	−	−
	++++	++++	++++	++++	++++	++	−	−	
Saline	−						−	−	
	−						−	−	

dispersed, but was aggregated into loose clumps. Boiling apparently does not lead to virus breakdown, and the fixing power of a boiled suspension is certainly due to particulate matter, since centrifugation for one hour on an angle centrifuge at 3000 r.p.m. deprives the supernatant fluid of the power to fix complement. It is difficult to believe, however, that the improvement in the complement-fixing power of the elementary body suspensions is merely a question of aggregation of virus particles. It seems more probable that boiling destroys something on the surface of the virus bodies and so unmasks the heat-stable antigen.

Absorption experiments.—There seems little doubt that psittacosis virus possesses a heat-stable antigen the nature of which is probably not protein. One would expect, however, that protein would form one of the main constituents of the virus, and that an antiserum made against the whole virus would therefore contain two antibodies. An attempt was made to investigate this possibility by means of absorption experiments. The material used for absorbing the serum was the deposit obtained by centrifuging a suspension of virulent mouse spleen on the angle centrifuge at 3000 r.p.m. for $1\frac{1}{2}$ hours. The suspension had been previously clarified by light centrifugation. When examined microscopically this deposit was seen to consist of elementary bodies mixed with a certain amount of fine tissue debris. It was resuspended in broth to the original volume of the crude suspension used and divided into two portions, one of which was boiled for one hour. The reason for using this deposit in preference to washed elementary bodies as antigen in these experiments was that the unheated elementary bodies had given such irregular results in previous complement-fixation experiments. Preliminary experiments were made to show whether specific absorption of antibody could be obtained, and if so, to give some idea of the relative quantities of serum and antigen which must be used to demonstrate this phenomenon. Absorption of 1·0 c.c. of the serum diluted 1 in 16 (complement-fixing titre 1 in 128 to 1 in 256) with the deposit obtained from 4·0 c.c. crude virus suspension was found to reduce the complement-fixing titre of the serum greatly, sometimes almost 100 p.c., whereas absorption with control material, obtained from a suspension of normal mouse spleen, produced no appreciable change in the titre of the serum. These absorptions were carried out in the refrigerator for about 18–20 hours, the absorbing material being removed at the end of that time by centrifugation. Several experiments have been carried out in which anti psittacosis serum, diluted 1 in 16, has been absorbed with the boiled and unboiled deposits obtained from crude virus suspensions, and the result of this on the complement-fixing titre for the crude and boiled antigens determined. Whereas absorption with unboiled material reduces the titre equally for the crude and boiled antigens, absorption with boiled material reduces the titre of the serum for itself, and has little or no effect on the power of the serum to react with crude antigen. An illustrative example is given in Table VII.

It would seem justifiable to conclude from these experiments that psittacosis virus possesses two antigens, one heat-labile and the other heat-stable, both of which give rise to antibodies capable of fixing complement specifically with their respective antigen.

TABLE VII.

Serum.		Antigens. Crude virus suspension.	Boiled psittacosis antigen.	Deposit from normal mouse spleen suspension. Unheated.	Deposit from normal mouse spleen suspension. Boiled.	Saline.
Unabsorbed	1/16	++++	++++	−	−	−
		++++	++++	−	−	−
	1/32	++++	++++			
		++++	++++			
	1/64	++++	++++			
		+++	+++			
	1/128	++	++			
		+	+			
	1/256	+	+			
		±	±			
Absorbed with with unheated antigen	1/16	+	+	−	−	−
		−	−	−	−	−
	1/32	−	−			
		−	−			
	1/64	−	−			
		−	−			
	1/128	−	−			
		−	−			
	1/256	−	−			
		−	−			
Absorbed with boiled antigen	1/16	++++	++++	−	−	−
		++++	+++	−	−	−
	1/32	++++	+			
		++++	±			
	1/64	++++	−			
		++	−			
	1/128	++	−			
		+	−			
	1/256	+	−			
		−	−			
Saline		−	−	−	−	−
		−	−	−	−	−

Allergic Reactions.

Guinea-pigs which have received psittacosis virus intradermally and reacted thereto are frequently found to be in an allergic state when re-inoculated two weeks later. This allergic state is best studied in albino guinea-pigs 500 g.

or more in weight. It is not always present, but has been noted as early as 10 days and as late as one month after the injection of living virus intradermally. How long it may persist is not known. In such an allergic animal the introduction of virus into the skin leads to the development of a raised red swelling some 3–4 cm. across, which reaches a maximum in 24 hours, and then rapidly fades so that by the third day there is little to be seen. In the normal animal, on the contrary, the reaction to virus inoculated intradermally takes three days to reach a maximum, and is then only about half the size of the fully developed allergic reaction.

Effect of heat on the antigen giving the allergic reaction.—Heating virus suspensions at temperatures ranging from 60° C. to 100° C. does not destroy the antigen which produces the allergic reaction in the sensitized guinea-pig, as is shown by the following experiment (Table VIII). The various products were inoculated in 0·2 c.c. quantities in the epilated skin of the flanks of a sensitized and of a normal guinea-pig.

TABLE VIII.

	Intradermal inoculation of 0·2 c.c. of a suspension of psittacosis virus.			
	Unheated.	Heated at 60° C. for 20 min.	Heated at 80° C. for 20 min.	Heated at 100° C. for 20 min.
Sensitized guinea-pig 1 day	+++	+++	+++	+++
Sensitized guinea-pig 3 days	+	+	+	+
Normal guinea-pig 1 day	+	—	—	—
Normal guinea-pig 3 days	++	—	—	—

+++ to — indicate the degree of reaction occurring at the site of inoculation.

It should be pointed out that the material used for inoculation, after the suspension had been heated at 80° C. and 100° C., was the clear supernatant fluid which was left after the proteins had coagulated. This supernatant fluid, it will be remembered, is quite incapable of giving fixation of complement, yet it apparently gives just as intense an allergic reaction as the crude suspension. An attempt quantitatively to compare the activity of the boiled extract and the crude suspension showed that, although the power to produce an allergic reaction was soon diluted out, having disappeared when the suspensions were diluted a hundredfold, both preparations gave parallel findings. Although results such as those recorded in Table VIII have usually been obtained, not every boiled extract has proved equally active. Evidence obtained from filtration experiments suggests that this irregularity is due to variations in the amount of antigen in solution in the different crude suspensions.

Effect of Seitz filtration on the antigen giving the allergic reaction.—It will be recalled that Seitz filtrates of crude virus suspensions failed to fix complement with a psittacosis antiserum and were devoid of virulence for the guinea-pig's skin. When tested for their power to produce an allergic reaction in the sensitized guinea-pig it was found that, as a rule, the filtrates were as active as the crude suspensions from which they were made. As with the boiled extracts, there were occasions when the filtrate did not give such good reactions as the parent suspension. Since both filtration and heating occasionally

had the effect of reducing the allergic potency of crude suspensions, it seems reasonable to interpret these irregularities as being due to variations in the degree of dispersion of heat-stable antigen in the crude suspensions. An attempt was made to find out what factors controlled the amount of highly dispersed heat-stable antigen in the crude suspensions. Wilson Smith (1933) found that the most potent boiled extracts of vaccinial material were obtained from suspensions which had been allowed to autolyse for some days at 37° C. The effect of this treatment, of varying periods of extraction in the cold and the use of different diluents—distilled water, saline, *M*/50 phosphate and broth—for making the suspensions were all investigated, but the results were inconclusive. Boiling, as was to be expected, had no effect on the power of a Seitz filtrate to give an allergic reaction.

Precipitin Reactions.

Attempts to obtain specific flocculation with crude virus suspensions or Seitz filtrates were abandoned owing to the fact that non-specific precipitation occurred so frequently with both these types of antigens. It was found, however, that the clear supernatant fluid obtained by boiling a crude suspension or a Seitz filtrate and removing the coagulated protein not only produced a specific allergic reaction in the sensitized guinea-pig, but gave also a precipitin reaction with a psittacosis antiserum. Though specific, this reaction was not a strong one; the greatest degree of precipitation obtained was visible to the naked eye, but in many instances a threefold magnification was necessary to detect the reaction with certainty. An example of such a precipitin test is given in Table IX.

TABLE IX.

Serum. Type.	Serum. Dilution.	Antigens. Boiled extract of— Virulent mouse spleen. 1/1.	1/2.	1/4.	Normal mouse spleen. 1/1.	Saline.
P. 264 anti-psittacosis	1/1	++	+	±	—	—
	1/2	±	∓	—	—	—
	1/4	—	—	—	—	—
P. 265 anti-psittacosis	1/1	++	+	±	—	—
	1/2	+	±	∓	—	—
	1/4	∓	—	—	—	—
Normal guinea-pig	1/1	—			—	—
Saline		—			—	—

++ = precipitate readily seen naked eye.
\+ = „ just visible naked eye.
± ∓ = „ seen only on 3 × magnification.
} Reading after 24 hours at 37° C.

Equally good results were obtained when the test was made at either 37° C. or 4° C., though 48 hours was required for the reaction to be complete at the latter temperature. In the water-bath at 55–56° C. no precipitation took place. Trying out this reaction with the serum from cases of human

psittacosis has shown that many of these give specific precipitation. But the reaction is not nearly so delicate as the complement-fixation reaction, and only those sera which fix complement strongly give a positive response to the precipitin test. Although the reaction has been found to be specific, in its present form it is not delicate enough to be used for diagnostic purposes. The reason for referring to this reaction here is that it provides additional evidence of a psittacosis antigen in the boiled extract. In a number of experiments the boiled extracts of crude suspensions were tested for ability to produce an allergic reaction and to form a precipitate with a psittacosis serum; they were also tested for the presence of protein by the addition of sulphosalicylic acid. The results (Table X) are interesting, since they show complete parallelism between the precipitin and allergic tests, and suggest that these reactions are independent of the traces of protein which are present in all these extracts.

TABLE X.

Boiled extract.	Precipitin test.	Allergic reactions.	Presence of protein.
No. 1156/7	—	—	±
,, 1162/3	+	++	±
,, 1165	+	++	±
,, 1170/1*a*	+	+	±
,, 1170/1*b*	—	—	±
,, 1170/1*c*	++	+++	±

DISCUSSION.

It would appear, then, that psittacosis virus possesses two antigens, one of which is resistant to boiling, whereas the other is rapidly destroyed at this temperature. Each of these antigens excites the production of specific antibody, and the reaction *in vitro* between these two antibodies and their respective antigens can be demonstrated by complement fixation. Crude virus suspensions possess a considerable amount of the heat-stable antigen unattached to virus particles, and since this free antigen is not carried down when the proteins in the crude suspension are coagulated by heat and is not removed by Seitz filtration, it must be in a high degree of dispersion and probably in solution. This free heat-stable antigen gives an allergic reaction in the skin of the sensitized guinea-pig and precipitates with a psittacosis antiserum, but it will not fix complement in the presence of a psittacosis antiserum, whereas the heat-stable antigen in the elementary bodies gives good complement-fixation. This does not mean, of course, that there are two different heat-stable antigens. It is more likely that they are the same, but behave differently in the complement-fixation reaction because of their different physical state.

The improvement in the complement-fixing power of washed elementary bodies is difficult to account for. It calls to mind the use of a "kokto-antigen" by Kraus and Takaki (1926) in their investigation of various rabies strains by the complement-fixation reaction. But whatever the explanation of the phenomenon may be, it would seem quite likely that a suspension of washed and boiled elementary bodies will prove to be a more satisfactory antigen than crude suspensions in the complement-fixation test used for the

diagnosis of human cases. The crude suspensions vary in their complement-fixing power and in the time during which they retain this property. Boiled virus would seem to give much more consistent results, and should keep much better than the crude antigens.

Of course, it remains to be seen whether antibody to the heat-stable antigen is regularly developed in human infections, but one or two observations have shown that when psittacosis antibody can be demonstrated by crude antigens, the reaction given with boiled antigen has been at least as good. No attempt has been made to find out what relationship exists between the two antibodies giving complement fixation and the neutralizing power of the serum. This omission is not due to any failure to appreciate the importance of this question, but to doubts as to the suitability of psittacosis antisera for this type of investigation. The neutralizing power of these sera is of such a low order that it seems doubtful whether one would obtain results sufficiently clear-cut to provide an unequivocal answer to this question ; other antiviral sera should be much more suitable for this type of work. However, a recently-made psittacosis antiserum has shown a much higher complement-fixing titre than any previous ones, and should it prove to have a reasonable neutralizing power, an attempt will be made with it to find out what connection neutralization has with the antibodies demonstrable by complement-fixation.

SUMMARY.

1. The complement-fixing power of a crude suspension of psittacosis virus depends on elementary bodies and more highly dispersed material, which is devoid of virulence, removed by Seitz filtration and thrown down to a certain extent by centrifugation at 10,000 r.p.m.

2. Psittacosis virus appears to contain two antigens, one resistant to boiling, the other rapidly destroyed at this temperature.

3. Psittacosis antisera contain antibodies to these two antigens, and union between them and their respective antigens *in vitro* can be demonstrated by complement fixation.

4. The clear supernatant fluid which is left when the protein in a crude suspension of psittacosis virus is coagulated by heat contains a heat-stable antigen, which gives an allergic skin-reaction in the sensitized guinea-pig and a weak precipitin reaction with a specific serum, but is of no use as an antigen in a complement-fixation test.

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THE DAELS AND BILTRIS TRANSPLANTABLE SARCOMA OF THE GUINEA-PIG.

A. F. WATSON.

From the Imperial Cancer Research Fund Laboratories, London.

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SPONTANEOUS neoplasms in the guinea-pig are rare. Maury (1931) mentions eleven tumours which have been recorded in the literature from time to time. Probably but few of these have proved to be transplantable. Until recently apparently the only such tumour in this country was Murray's (1916) liposarcoma. This occurred originally in an animal from the Lister Institute stock, and has been maintained here continuously for twenty years without altering its characteristics. In describing this tumour, which in many ways is a remarkable one, Murray states :

> "It is probable that further experience will necessitate its association with the malignant new growths under the designation of liposarcoma. . . . Biologically this sarcoma of the cavy is characterized by an extraordinary slow rate of growth. Nodules of palpable size seldom appear before a month has elapsed after inoculation, and thereafter increase in size is very gradual. Consequently a very perfect vascularization of the new tissue and adaptation of the organism to its presence takes place, so that enormous tumour masses are produced and supported without inconvenience. . . . Success of implantation has varied from 30 to 100 p.c. and averages 50 p.c. . . . Spontaneous regression is rare. . . . Ulceration rarely occurs. . . . In spite of the long periods for which it has already grown in individual animals, macroscopic metastases have not been observed in the lungs or lymphatic glands. However, microscopic examination of the lungs of a cavy of the seventh generation in which a tumour had grown for eight months and attained a weight of 340 gm. showed the presence of an embolus of living tumour cells in a small branch of a pulmonary artery."

For some years Daels and his colleagues have studied tumours produced by the prolonged action of radium in different species of animals, including the guinea-pig (Daels and Biltris, 1931 ; Biltris, 1933). Through the courtesy of Professors Daels and Biltris we have been able to study one of these cavy tumours, a sarcoma which is readily transplantable. The present paper describes the behaviour of this tumour, which has been maintained here under laboratory conditions for over a year.

The following table, which is self-explanatory, summarizes the characteristics of and emphasizes the differences between the Daels and Biltris tumour and the Murray tumour. Metastases frequently occur in the former and are much more common than with transplantable tumours of rats and mice.

Fig. 1 shows the primary tumour with widespread metastases in an animal seventy days after transplantation of the original tumour fragment. High-power views of the primary tumour and of a heart metastasis (which is

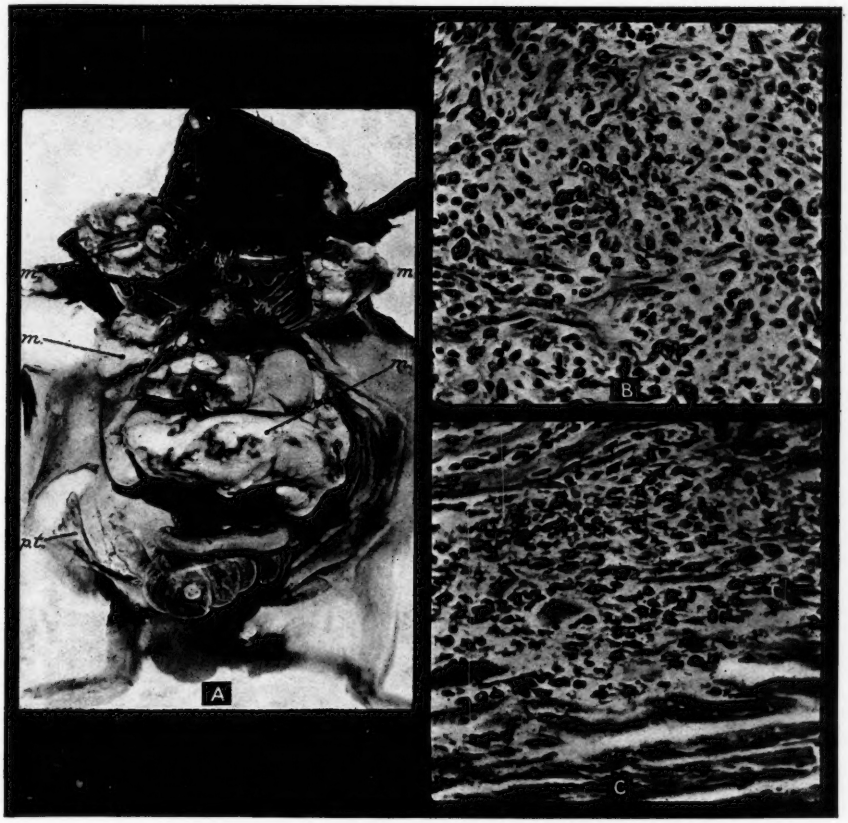

FIG. 1.—A. Primary tumour and metastases, 70 days; B. Primary tumour, × 275. C. Heart metastasis, × 250.

extremely rare in the case of rat or mouse transplantable tumours) are also shown.

Comparative Behaviour of the Murray Liposarcoma and the Daels and Biltris Sarcoma of the Guinea-pig.

	Murray tumour.	Daels and Biltris tumour.
Type	Liposarcoma	Sarcoma.
Origin	Occurred spontaneously	Induced by radium.
Tumour "takes" p.c.:		
Range	30–100	70–100
Average	50	80
Rate of growth	Very slow	Vigorous.
Regression	Very rare.	Rare.
Ulceration	,,	Frequent.
Metastases	Macroscopically never seen. Groups of living tumour-cells sometimes seen in lung sections.	Frequent in animals with large primary tumours.
Chemical reducing activity (of type similar to vitamin C) of fresh tumour	About half that of rat and mouse tumours. Depends on vitamin C content of diet	About half that of rat and mouse tumours. Depends on vitamin C content of diet.

SUMMARY.

The behaviour under laboratory conditions of the Daels and Biltris transplantable sarcoma of the guinea-pig is described and contrasted with that of the Murray liposarcoma. Its most interesting features are its vigorous growth, the frequency with which it metastasises, and, compared with rat and mouse tumours, its low content of reducing substances of a type similar to vitamin C.

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THE CHEMICAL REDUCING CAPACITY AND VITAMIN C CONTENT OF TRANSPLANTABLE TUMOURS OF THE RAT AND GUINEA-PIG.

A. F. WATSON.

From the Imperial Cancer Research Fund Laboratories, London.

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TRICHLORACETIC acid extracts of tumours, like those of normal tissues, show a reducing activity of a type similar to that of ascorbic acid (vitamin C) (Boyland, 1933; Harris, 1933*a*; Watson and Mitolo, 1934). The evidence as to whether such activity is due to vitamin C or to some other substance is so far conflicting. Harris and Ray (1932) concluded that the reducing activity of the ox suprarenal could be accounted for by its biologically determined vitamin C content, and Birch and Dann (1934) showed the presence of ascorbic acid in the eye lens and aqueous humour of the same animal by feeding tests. Young and Mitolo (1934), on the other hand, were of the opinion that the high reducing activity of brain tissue is not due to its ascorbic acid content. In the case of tumours, Harris (1933*b*), in a preliminary communication, was unable to associate the reducing activity of the Jensen rat sarcoma entirely with its vitamin C content, and suggested the existence of a hitherto unrecognized powerfully reducing substance. Borghi and Deotto (1934) similarly failed to establish the association in the case of the Ehrlich mouse carcinoma. Bierich (1934) has stated that tumour tissue contains vitamin C.

The possible *rôle* of vitamin C either in carcinogenesis or in the actual metabolism of tumours has so far received but scant attention. The recent identification of the vitamin with ascorbic acid has stimulated studies in this field. Woodhouse (1934) has claimed that this acid, when injected into mice, is capable of accelerating the rate at which tar warts show invasive characteristics. Now, in their relationship to ascorbic acid, mice and rats, on the one hand, differ from the guinea-pig, monkey and man on the other. While the former are able to synthesize the acid and therefore can dispense with its presence in the diet, the latter are completely dependent on the diet for this essential substance. Accordingly, in laboratory studies on the relationship of ascorbic acid to tumours, the guinea-pig possesses advantages over the rat and mouse both in its dependence on the diet for the supply of the acid and in its resemblance to man in this important respect. Apart from the Murray liposarcoma, which is of limited use for experimental work, owing to its extraordinary slow rate of growth, no suitable cavy tumour has hitherto been available. The recent production by Daels and Biltris of the readily transplantable cavy tumour described in the previous paper (Watson, 1936) has provided an opportunity of studying the relationship of ascorbic acid to tumour metabolism in an animal which, for many years, has been used for vitamin C

experiments. The present paper is concerned with the chemical reducing capacities and antiscorbutic activities of transplantable tumours of the guinea-pig compared with those of the rat.

METHODS.

The following transplantable tumours were used :

(1) The Jensen rat sarcoma and, less frequently, the Walker rat carcinoma.

(2) The Daels and Biltris cavy sarcoma and, less frequently, the Murray cavy liposarcoma.

Dried tumours.—Freshly excised tumours were freed from necrotic portions, minced finely and dried down rapidly over sulphuric acid *in vacuo*. The dried tissue was ground lightly to a coarse powder.

Expressed tumour juices were prepared as follows : Fresh tumour tissue was minced and mixed intimately with sterile sand. The mixture was placed in the autoclaved cylinder of a hydraulic press and subjected to a pressure of about two tons to the square inch. The expressed liquid was collected and centrifuged, or, in the case of some tumours, was filtered free from solid particles.

The chemical reducing activities of tissues.—The method employed for estimating the reducing activities of normal and malignant tissues was similar to that used before (Watson and Mitolo, 1934), and is a slight modification of that described by Birch, Harris and Ray (1933). For convenience the reducing value (R.V.) of a tissue has been expressed in terms of the number of c.c. of a 1/10,000 dilution of the indicator dichlorophenolindophenol reduced by the substances extracted from one gramme of tissue by trichloracetic acid under the conditions of the method.

The antiscorbutic activities of malignant tissues.—The curative test with scorbutic guinea-pigs was used for the estimation of the vitamin C content. This gives fairly accurate comparative results in a short time and demands only moderate amounts of material. The quantitative administration of fresh tumour tissue and the injection of tumour extracts or juices have proved less satisfactory than the administration of tumour desiccates. The technique of the curative test was as follows.

Guinea-pigs (usually males weighing 200–300 g.) were placed on Bracewell, Hoyle and Zilva's (1930) scorbutic diet supplemented for about a week with fresh cabbage. The latter was then withdrawn from the diet and the animals subsequently weighed at short intervals until the third week, when those animals which had shown normal weight increases with the characteristic drop at this time (indicating the onset of scurvy) were selected for the tests. Weighed amounts of the different dried tumours were mixed with a small amount of the basal diet and fed to the animals early each morning. The desiccates were standardized for reducing activity at the beginning and the end of the feeding tests. As soon as the daily supplement had been consumed the animals were allowed access to unlimited amounts of the basal diet. Daily weighings were continued for 10–20 days. At the end of this time the guinea-pigs were killed and the reducing values of some of the tissues (usually the adrenals and liver) estimated. The teeth and ribs were prepared for histological examination. As controls some animals were given no tumour supplements, and others were injected with freshly-prepared solutions of ascorbic acid at the rate of 1 mg. per day. The usual method of employing several animals for each of the varying amounts of supplement tested could not be used owing to the large amounts of dried tumour

necessary. Recourse was therefore made to the employment of one or two animals only for each amount of desiccate tested. Such experiments were repeated several times with fresh batches of dried tumours.

The ribs and teeth were fixed in Zenker formol, washed and then decalcified in 5 p.c. sulphurous acid. Frozen sections were cut and stained with Ehrlich's hæmatoxylin and eosin. No photographs of the teeth sections are included below, but these confirmed the effects illustrated in the rib sections.

THE CHEMICAL REDUCING VALUES OF TISSUES.

The Chemical Reducing Values of Transplantable Tumours of (1) *Rats and of* (2) *Guinea-pigs allowed Access to Ample Vitamin C.*

Expressed tumour juices, trichloracetic acid extracts and desiccates of tumours show a high concentration of reducing substances. There is a higher concentration in the rat tumours than in the cavy tumours (Table I). An appreciable loss of reducing activity takes place when the tumours are dried *in vacuo* over sulphuric acid. This loss, however, may be more apparent than real if, as is possible, the acid extraction of the desiccates is less complete under the conditions of the test than the extraction of the fresh tissues. The analyses of a number of batches of dried tumours showed that these contain reducing substances equivalent to about 1·1–1·5 mg. (in the case of the Jensen rat tumour) and about 0·35 mg. or less (in the case of the Daels and Biltris cavy tumour) ascorbic acid per g. *In vacuo* the desiccates usually retain their reducing activity for some weeks at least.

TABLE I.—*The Relative Reducing Capacities of Transplantable Tumours of Rats and Guinea-pigs.*

	Rat.		Guinea-pig.	
	Jensen sarcoma.	Walker carcinoma.	Murray liposarcoma.	Daels and Biltris sarcoma.
Expressed tumour juices :				
c.c. of expressed juice equivalent in reducing activity to 1 mg. of ascorbic acid	2·8	3·1	9·4	7·5
Trichloracetic acid extracts :				
Mg. of ascorbic acid equivalent in reducing activity to trichloracetic acid extracts from 1 g. of fresh tissue	·35–·48	·38–·56	·14–·24	·17–·21
Tumour desiccates :				
Dry weight of tumour p.c.	17·4	..	..	12·5–14·0
Mg. of ascorbic acid equivalent in reducing activity to trichloracetic acid extracts from 1 g. of dried tissue.	1·1–1·5	..	Variable, ·35 or less	Variable, ·35 or less.

The chemical reducing values of both normal and malignant tissues of the rat, which is able to synthesize vitamin C, remain comparatively constant whether the vitamin (ascorbic acid) is supplied to the animal or not. Such is not the case, however, with the guinea-pig, which is unable to synthesize the vitamin. This is shown in the following series of experiments.

The Chemical Reducing Values of Normal and Malignant Tissues of Guinea-pigs Deprived of and Subsequently Supplied with Ascorbic Acid in Varying Amounts.

If guinea-pigs are transferred from a diet rich in vitamin C to one containing none (*e. g.* Bracewell, Hoyle and Zilva's (1930) scorbutic diet), the reducing values of both normal and malignant tissues gradually decrease.

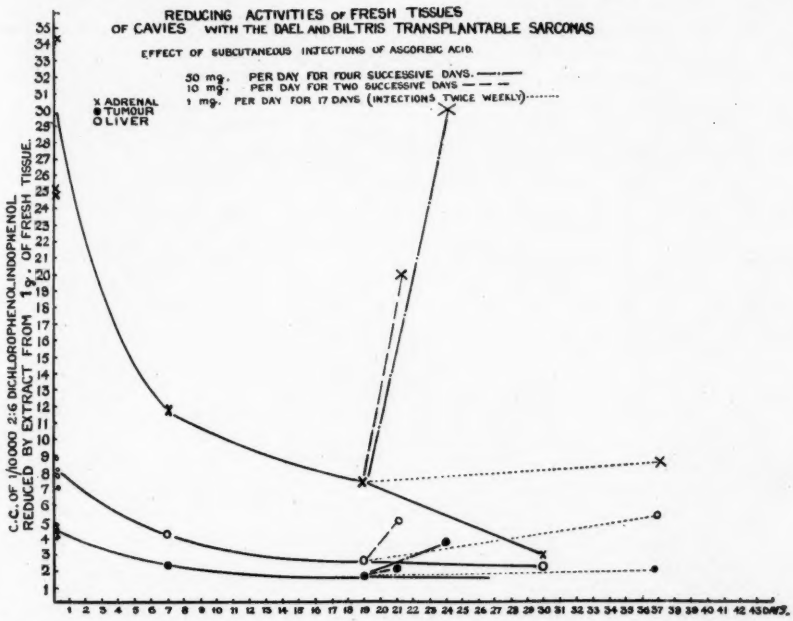
REDUCING ACTIVITIES OF FRESH TISSUES
OF CAVIES WITH THE DAEL AND BILTRIS TRANSPLANTABLE SARCOMAS
EFFECT OF SUBCUTANEOUS INJECTIONS OF ASCORBIC ACID.
50 mg. PER DAY FOR FOUR SUCCESSIVE DAYS.
10 mg. PER DAY FOR TWO SUCCESSIVE DAYS.
1 mg. PER DAY FOR 17 DAYS (INJECTIONS TWICE WEEKLY)
× ADRENAL
● TUMOUR
○ LIVER
C.C. OF 1/10000 2:6 DICHLOROPHENOLINDOPHENOL REDUCED BY EXTRACT FROM 1 g. OF FRESH TISSUE.

FIG. 1.—The withdrawal of vitamin C from the diet of tumour-bearing guinea-pigs was followed by a gradual loss of reducing substances from the normal and malignant tissues. These reducing substances were restored if ascorbic acid was injected subcutaneously into the animals before the scorbutic symptoms became too severe. With small amounts of ascorbic acid the recoveries of the reducing values of normal and malignant tissues were only partial.

This is shown in Fig. 1, where the decreases in the adrenals, liver and tumour (the Daels and Biltris sarcoma) are recorded during the thirty days following the withdrawal of the vitamin. Growth of the animal and of its tumour continue during the first 17–20 days, although by this time the reducing values of the tissues have reached low levels. Subsequently a steady loss of weight occurs, the death of the animal taking place about the 27th–30th day, with typical scorbutic symptoms.

The histological changes in the teeth and ribs under such conditions of vitamin C deficiency are well known. Wolbach and Howe (1926) and Menkin, Wolbach and Menkin (1934) maintain that the important change in the teeth is the cessation of dentine formation and the separation

of the odontoblast layer from the dentine. At the costochondral junctions of the ribs osteoblastic proliferation continues uninterruptedly, without, however, producing any bone matrix. Scurvy is visualized as primarily due to a cessation in the normal formation of intercellular material by supporting tissues. Fish and Harris (1934) consider that a deficiency of vitamin C affects the vitality of all cells which are specially concerned with the laying down of the hard tissues. Primarily there is simply premature degeneration and death of the odontoblasts and ameloblasts associated with degeneration of the cementoblasts, osteoclasts, osteoblasts and bone-corpscles. In the most severe cases there is degeneration of the general connective tissue of the dental pulp, with congestion of its vessels. All other changes are regarded as secondary.

If at the 17th–20th day of the diet the partially scorbutic animals are injected subcutaneously with ascorbic acid, the progress of the disease is interrupted. The effect of such treatment on the recovery of the reducing values of some of the tissues (adrenals, liver and tumour) is shown in Fig 1. Table II shows, in addition, the degree of repair in the teeth and ribs and the weight increases of the animals in the same experiments.

The results of these studies where ascorbic acid has been injected under varying conditions into partially scorbutic guinea-pigs may be summarized as follows : (1) Advanced reparative processes in the teeth and bones, a steady weight recovery, and a *partial* recovery of the reducing values of the normal and malignant tissues (as illustrated by the adrenals, liver and tumour) were effected by the administration of ascorbic acid twice weekly at the rate of 1 mg. per day for from 9–17 days. (2) The initiation of reparative processes in the teeth and bones, and a *partial* recovery of the reducing values of the adrenals, liver and tumour, were obtained by the administration of 10 mg. of ascorbic acid per day for two days. (3) The initiation of reparative processes in the teeth and bones, some increase in the weights of the animals and a *complete* recovery of the reducing values of the adrenals and tumour to their optimal levels were effected by the administration of 50 mg. of ascorbic acid per day for four days.

These results appear to justify the following conclusions : (1) Whether the reducing activities of normal and malignant tissues are due entirely or only partially to ascorbic acid, or alternatively to some other substance or substances, such activities must be related (at any rate in these experiments*) to the ascorbic acid supplied to the guinea-pigs. (2) The initiation of reparative processes in the teeth and bones, and the subsequent resumption of growth in partially scorbutic guinea-pigs, are induced by amounts of ascorbic acid which are less than those necessary to restore the reducing values of the normal and tumour tissues to their optimal levels.

How far the various tissues and in particular the malignant tissues owe their reducing capacities to the presence of ascorbic acid can only be determined by biological assay. To investigate the malignant tissues, the administration of fresh tumours to, and the injection of tumour juices into, partially scorbutic cavies were first attempted. Such attempts were usually unsatisfactory, and recourse was made eventually to the oral administration of tumour desiccates of known chemical reducing activities. As controls the responses of partially scorbutic guinea-pigs to the injection of ascorbic acid were studied. These control experiments will be described first.

* Mitolo (1934) has recently stated that the tissues of pigeons kept on a polished rice diet show a similar diminution of reducing substances.

TABLE II.—*The Reducing Values of Normal and Malignant Tissues, the Degree of Repair in Ribs and Teeth and the Growth of Guinea-pigs on a Scorbutic Diet after Injections of Varying Amounts of Ascorbic Acid.*

Series.	Number of days on scorbutic diet.	Amount and duration of ascorbic acid injections.	Reducing values of tissues.			Degree of repair.		Increase in weight after injections (g.).	Remarks.
			Adrenal.	Liver.	Tumour.	Ribs.	Teeth.		
..	19	No injections	<2·0	<2·0	<2·0	..	..	..	Fig. 3A.
		Twice weekly injections at rate of 1 mg. per day :							
11 (2)	17	(*a*) for 9 days	7·4	4·1	..	+++	+++	10	Figs. 2, 3B.
16 (2)	19	(*b*) „ 16 „	11·3	2·0	1·6	++++	++++	67	Fig. 2.
10 (6)	20	(*c*) „ 17 „	8·5	5·3	..	++++	++++	56	Figs. 1, 2 and 3C.
11C (2)	19	10 mg. per day for 2 days ; killed 3rd day	20·0	5·2	2·0	+	+	..	Fig. 1.
19 (1)	20	50 mg. per day for 4 days ; killed 5th day :	30·0	..	3·5	+	+	20	Fig. 1.
19 (2)	20		33·4	..	..	+	+	5	..

Optimal reducing values (animals allowed ample vitamin C.) : Adrenal, 25–36 ; liver, 7–9 ; tumour, 3·5–5·0.

THE ANTISCORBUTIC ACTIVITIES OF DRIED RAT AND GUINEA-PIG TUMOURS COMPARED WITH THAT OF ASCORBIC ACID.

The Response of Guinea-pigs on a Scorbutic Diet to the Subcutaneous Injections of Ascorbic Acid.

Svirbely and Szent-Györgyi (1932) found that 1 mg. per day of ascorbic acid administered with the diet afforded a guinea-pig complete protection against scurvy in a 90 days' test. This amount is probably near the minimum necessary to provide this protection. As an alternative to feeding, the ascorbic acid may be administered parenterally. The weight curves of

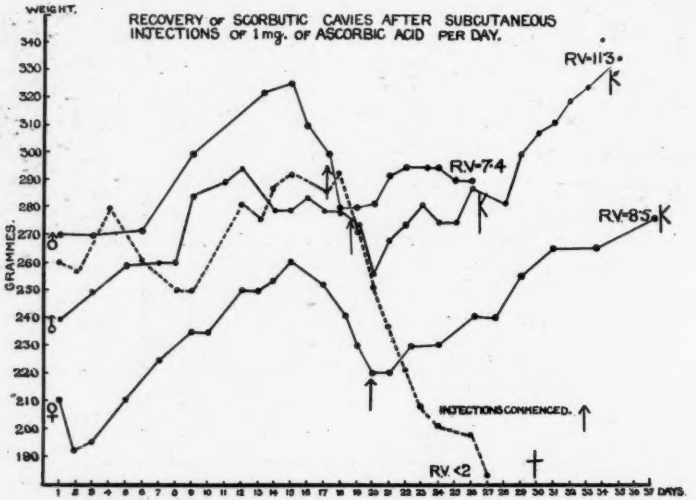

FIG. 2.—The progressive loss of weight of guinea-pigs after 2–3 weeks on a scorbutic diet was arrested and followed by a steady weight recovery when the animals were injected with ascorbic acid twice weekly at the rate of 1 mg. per day. The adrenal reducing values (R.V.), however, showed only a partial recovery to about 33 p.c. of their optimal value at the end of the treatment.

three animals on a scorbutic diet for 17–20 days before the subcutaneous injection of ascorbic acid twice weekly at the rate of 1 mg. per day are shown in Fig. 2. The animals were injected at different times and for varying periods. A fourth control animal which received no ascorbic acid died on the 30th day.

The injected animals showed: (1) An early arrest in the previously progressive loss of weight, with subsequent steady growth. (2) Typical bone repair at the costochondral junctions of the ribs (Fig. 3, B and C), and renewed dentine formation in the teeth. (3) A partial recovery (to about 33 p.c. of the optimal levels) of the adrenal reducing values (Fig. 1 and Table II).

The studies were continued from this point to determine the lowest amounts of dried rat and guinea-pig tumours which, when fed to partially scorbutic

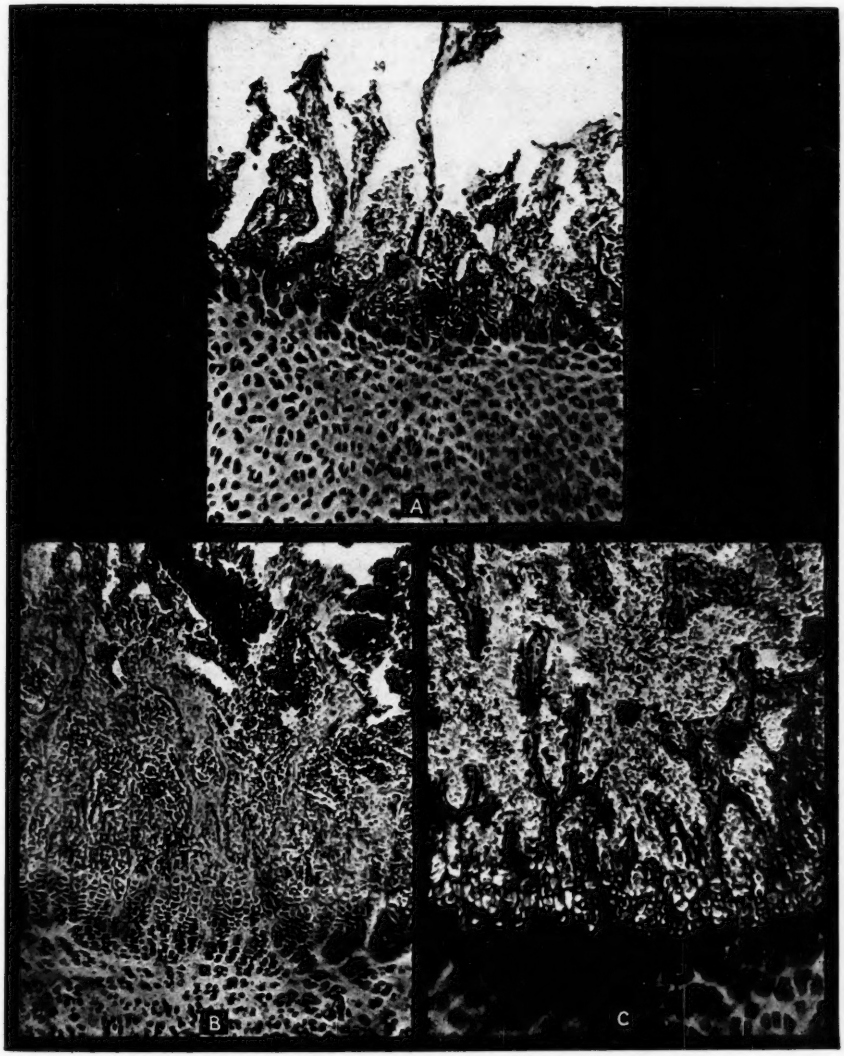

FIG. 3.—The effect of ascorbic acid on the progress of scurvy in the guinea-pig. A. The withdrawal of vitamin C from the diet of a guinea-pig. Costochondral junction of rib × 70. Failure of the repair process after the animal had been 19 days on a scorbutic diet. B and C. The effect of the subcutaneous injections of ascorbic acid twice weekly at the rate of 1 mg. per day (B) for 9 days, and (C) for 17 days after (B) 17 days and (C) 20 days on a scorbutic diet. Costochondral junctions of the ribs × 70. Initiation of the repair process.

guinea-pigs, would induce a similar response to the injections of ascorbic acid at the rate of 1 mg. per day.

The Response of Guinea-pigs on a Scorbutic Diet to the Oral Administration of Dried Jensen Rat Sarcoma.

The effect of supplementing the diet of 18-day scorbutic guinea-pigs with 0·5, 1, 2 and 3 g. respectively per animal per day of dried Jensen rat sarcoma is shown in Fig. 4. There was good weight recovery of the animals on the three largest supplements, although that of the animal on the 1 g. daily supple-

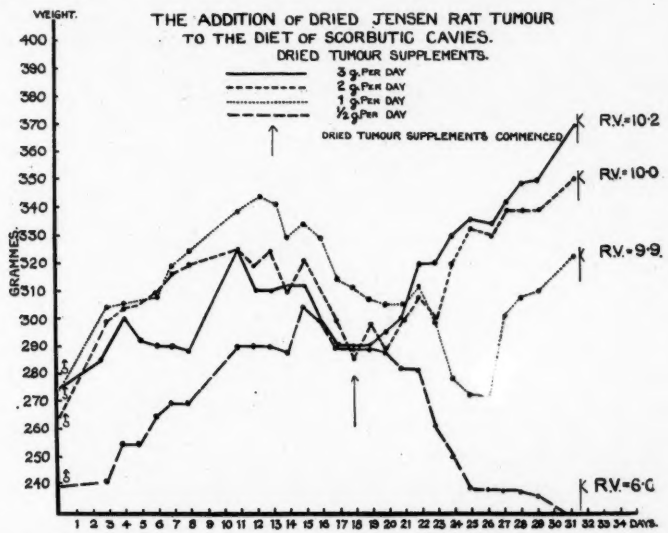

FIG. 4.—The progressive loss of weight of guinea-pigs after 2–3 weeks on a scorbutic diet was here again arrested if the diet of the animals was supplemented with 1, 2 or 3 g. per day of dried Jensen rat sarcoma. Here, again, the recovery of the adrenal reducing values was only partial at the end of the 14 days' treatment. The animal on the 1/2 g. daily supplement of dried tumour showed no weight recovery, but exhibited a partial recovery of the adrenal reducing value.

ment was somewhat delayed. There was no arrest in the progressive loss of weight of the animal on the 0·5 g. daily supplement, and a fifth control animal (curve not shown) on an unsupplemented diet died on the 30th day with typical scorbutic symptoms.

There was a gradually increasing repair reaction in the ribs (Fig. 5) and in the teeth. Even in the animal on the 0·5 g. daily supplement (which showed no arrest in the loss of weight) some bone and teeth repair was found. The control animal which received no dried tumour showed the typical failure of the reparative processes characteristic of the 30-day scorbutic guinea-pig (Fig. 5 A).

Despite the resumption of growth of the animals on the largest supplements, the reducing values of the tissues did not show complete recovery.

During the period of the experiment they did not attain, for instance, in the case of the adrenals, the optimal value of 25–36. The adrenal value for the animal on the unsupplemented diet was less than 2·0. Those of the animals on the diets supplemented with 0·5, 1, 2 and 3 g. daily were 6·0, 9·9, 10·0 and 10·2 respectively (Fig. 4). The 6·0 value for the animal on the smallest supplement was somewhat unexpected in view of the animal's failure to resume growth. Some repair was evident in the ribs and teeth of this animal.

These experiments show that a daily supplement of 1 g. of dried Jensen rat sarcoma to the diet of scorbutic guinea-pigs was the smallest amount capable of effecting a response similar to that found after injecting ascorbic acid in amounts of 1 mg. per day. Now 1 g. of this dried tumour has been shown to contain reducing substances equivalent to from 1·1 to 1·5 mg. of ascorbic acid. The antiscorbutic activity of the desiccate, therefore, approaches that demanded by its reducing activity, if this is due entirely to ascorbic acid. The nature of the reducing substances lost on desiccation of the tumour cannot be assumed without further investigation to possess antiscorbutic activity. Whether tumour tissue, as has been suggested (*e. g.* by Harris, 1933*b*), contains, in addition to ascorbic acid, a hitherto unrecognized substance of high reducing activity is left uncertain by these studies.

The Response of Guinea-pigs on a Scorbutic Diet to the Oral Administration of Dried "Daels and Biltris" Cavy Sarcoma.

The Daels and Biltris cavy tumour desiccates usually contain reducing substances equivalent to about 0·35 mg. of ascorbic acid per g. If the reducing activity were due entirely to ascorbic acid, about 3 g. of the material per day should be sufficient to effect the cure of scorbutic guinea-pigs under the conditions employed in the previous studies. In a limited number of tests, the administration of amounts up to 6 g. per day has so far failed to arrest the progressive loss of weight of the animals (Fig. 6), although a certain amount of repair in the teeth and ribs has been evident. Similar results have been found in a few tests with the Murray cavy liposarcoma.

Where supplements of *fresh* cavy tumours have been fed *ad lib.* to scorbutic animals, the more gross scorbutic symptoms have been postponed, and increased tissue-reducing values induced. No steady weight recoveries of the animals have been obtained however. While these cavy tumours probably contain vitamin C, although in a smaller concentration than the rat tumours, it is difficult to decide whether the chemical reducing values are an index of the amount of the vitamin. The success of feeding experiments of this type depends on whether or not it is possible to administer to guinea-pigs quantitatively supplements which contain known amounts of reducing substances. While it is a simple matter to do this when the active substances are contained in a small amount of dietary supplement, it becomes increasingly difficult when, with lower concentrations of reducing substances, it is necessary to increase considerably the size of the supplements. The animals must be persuaded to consume the supplement, and they must consume it before the activity of the vitamin, if present, is decreased or destroyed.

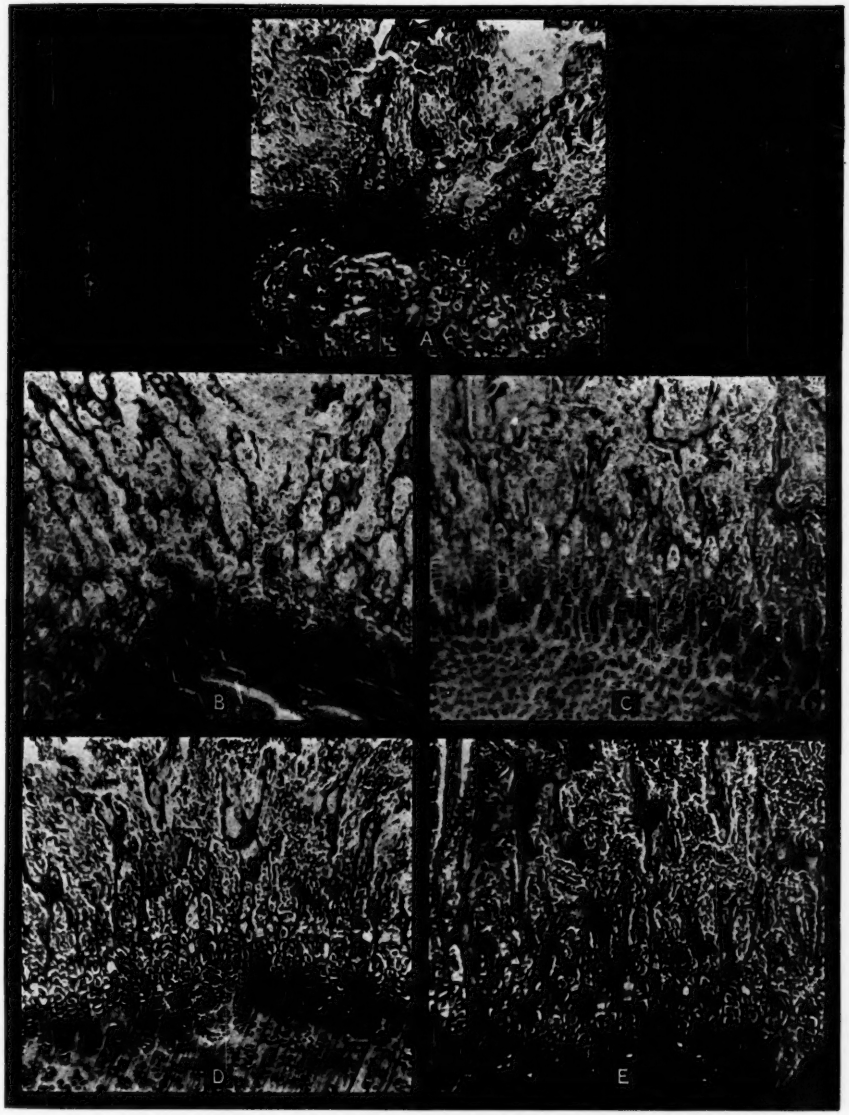

FIG. 5.—The effect of the oral administration of dried Jensen rat sarcoma on the progress of scurvy in the guinea-pig. The animals were given the scorbutic diet for 18 days, subsequently supplemented with (A) 0 g., (B) 1/2 g., (C) 1 g., (D) 2 g. and (E) 3 g. of dried Jensen rat sarcoma per day for 14 days. Costochondral junctions of ribs × 70. There is a gradual increasing repair reaction with the increasing supplements of dried tumour.

Watson.

There is indirect evidence that in the case of the guinea-pig, tumour-cells, like normal cells, utilize vitamin C. Animals with rapidly-growing Daels and Biltris tumours and maintained on a diet deficient in vitamin C usually have a shorter life-span than similarly fed animals without tumours (Table III).

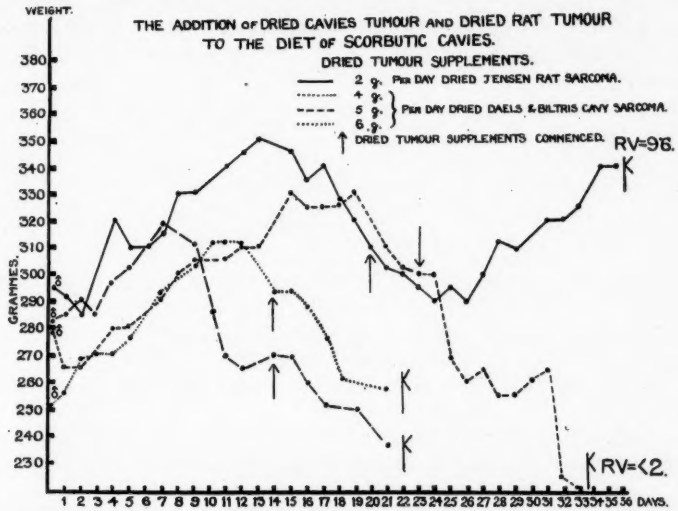

FIG. 6.—The progressive loss of weight of guinea-pigs after 2–3 weeks on a scorbutic diet was not arrested when the animals were given dried "Daels and Biltris" cavy sarcoma in amounts varying from 4 to 6 g. per day. As before, 2 g. of Jensen rat sarcoma dried in the same way as the cavy tumour induced a steady weight recovery. R.V. = Reducing values of adrenals at end of experiment.

TABLE III.—*The Survival Periods of Guinea-pigs* (a) *Without Tumours and* (b) *With Rapidly-growing Daels and Biltris Tumours, after the Withdrawal of Vitamin C from the Diet.*

	Survival periods of animals (days).	Average survival period.
Animals without tumours (— C diet).	12, 13, 20, 21, 26, 26, 27, 28, 28, 33, 33	24·3 days.
Animals with rapidly-growing tumours (— C diet)	7, 10, 10, 12, 12, 13, 13, 16, 16, 16, 20	13·2 ,,
Animals with rapidly-growing tumours (+ C diet)	..	>30 ,,

If the vitamin C in the diet is discontinued, the animals survive only as long as the reserves in the tissues last. These reserves become exhausted more quickly if the animals are supporting rapidly-growing tumours. The latter, like normal tissues, must be making demands on the available vitamin C supply.

SUMMARY.

(1) A progressive loss of reducing substances from normal and malignant tissues occurs when vitamin C (ascorbic acid) is withdrawn from the diet of guinea-pigs for from two to three weeks. These substances can usually be restored during this period by the administration of ascorbic acid in adequate amounts. Restoration to optimal values demands larger amounts of ascorbic acid than are sufficient to induce resumption of growth with typical repair in the teeth and ribs.

(2) The Jensen rat sarcoma dried *in vacuo* over sulphuric acid contains measurable amounts of vitamin C which can be determined biologically. Two transplantable cavy tumours when dried under similar conditions showed only slight signs of vitamin C activity, even when fed at much higher level than the rat tumour. With these differences must be correlated the lower chemical reducing values of the cavy tumours compared with those of the rat.

(3) Within the limits of error of the tests, the antiscorbutic activity of dried Jensen rat sarcoma (as determined by feeding tests) approaches that demanded by its reducing activity if this is due to ascorbic acid. There is an appreciable loss of reducing substances on desiccation of the tumour and the nature of these is not certain. Whether tumours contain in addition to ascorbic acid a hitherto unrecognized substance of high reducing activity remains undecided.

(4) The vitamin C reserves in the tissues of guinea-pigs on a scorbutic diet are exhausted more rapidly if the animals are supporting rapidly-growing tumours. Like normal cells, tumour-cells utilize vitamin C.

I am indebted to Dr. W. Cramer for many suggestions during the course of these studies.

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EXPERIMENTS ON THE FILTRATION OF CLIMATIC BUBO (LYMPHOGRANULOMA INGUINALE) VIRUS THROUGH "GRADOCOL" MEMBRANES.

J. C. BROOM AND G. M. FINDLAY.

From the Wellcome Bureau of Scientific Research, London.

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DURING the last three years filtration experiments through gradocol membranes have from time to time been carried out with the virus of climatic bubo (lymphogranuloma inguinale). A number of observers have shown that this virus is able to pass through Berkefeld V and N filters, Chamberland L_1, L_2, L_3 candles and Seitz E and K filters, while Miyagawa, Mitamura, Yaoi, Ishii and Okanishi (1935) have found that the virus can also pass through collodion membranes with pore sizes of 0·33μ, but not with pore sizes of 0·24μ. All the experiments here recorded were carried out with infected mouse brain. Four strains of the virus were employed, one of them from a case infected in this country. Brains were removed from animals showing the characteristic clinical symptoms and ground up in Hartley's broth to form a 10 p.c. suspension. This suspension was centrifuged for 15 minutes at 4,000 r.p.m. and the supernatant fluid filtered through a sand and pulp filter (Barnard and Elford, 1931).

Technique.—As in previous experiments on the filtration of Rift Valley fever virus (Broom and Findlay, 1933) and yellow fever virus (Findlay and Broom, 1933), the methods described by Elford (1931 and 1933) for the preparation and use of collodion filters of regular and graded pore size have been followed. Twelve mice were inoculated intracerebrally with the filtrates, the same number of controls being inoculated with material from the sand and pulp filter. As cerebral symptoms in mice develop somewhat irregularly, all inoculated mice were kept under observation for at least two months. The brains of mice dying during this period were examined histologically to determine the presence of the lesions characteristic of lymphogranuloma inguinale infection. At the end of two months all mice that remained alive were killed and their brains sectioned.

The results are shown in the table. It will be seen that virus passed through membranes with pore sizes of 0·35μ, but not through those with pore sizes of 0·25μ and 0·15μ. Elford (1933) has calculated that membranes of pore size 0·1–0·5μ retain particles of from 0·5 to 0·75 the diameter of the pores. The dimensions of the virus particles of lymphogranuloma inguinale are thus from 0·125μ to 0·175μ—a size similar to that of the vaccinia virus as estimated by Elford and Andrewes (1932). This result thus resembles that obtained by Miyagawa and his colleagues (1935).

Filtration of Climatic Bubo Virus.

Average pore size of membrane (μ).	Thickness (mm.).	Filtration pressure (cm. Hg).	Volume filtered (c.c. per sq. cm.).	Infectivity of filtrate (0·03 c.c. intra-cerebrally).	Infectivity of stock mouse brain suspension (0·03 c.c. intracerebrally).
0·55	0·16	20	5	5 of 12 mice positive	8 of 12 mice positive.
0·55	0·16	20	5	6 „ 12 „ „	6 „ 12 „ „
0·55	0·16	20	5	5 „ 12 „ „	8 „ 12 „ „
0·35	0·20	20	10	4 „ 12 „ „	8 „ 12 „ „
0·35	0·20	20	10	2 „ 12 „ „	7 „ 12 „ „
0·25	0·17	40	10	0 „ 12 „ „	6 „ 12 „ „
0·25	0·17	40	10	0 „ 12 „ „	8 „ 12 „ „
0·15	0·16	40	10	0 „ 12 „ „	5 „ 12 „ „
0·15	0·16	40	10	0 „ 12 „ „	8 „ 12 „ „

SUMMARY.

By the use of "gradocol" membranes the size of the virus of climatic bubo (lymphogranuloma inguinale) is estimated to lie between 0·125μ and 0·175μ.

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THE COLI-TRYPTOPHAN-INDOLE REACTION: II. THE NON-PRODUCTION OF TRYPTOPHANASE IN MEDIA CONTAINING GLUCOSE.

FRANK CHARLES HAPPOLD AND LESLIE HOYLE.

From the Department of Pathology and Bacteriology, the University of Leeds.

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IN a previous paper the present authors (1935) described the preparation of non-viable suspensions of *B. coli*, which would convert tryptophan into indole in the presence of oxygen. The name "trytophanase" was given to these preparations. They were shown to be active between pH 5·0 and pH 10·0, with an optimum at pH 8·5. A subsequent point of interest was the discovery that the enzyme was produced, if weakly, in a synthetic medium free from

all traces of tryptophan. We also showed that our tryptophanase preparations were incapable of converting a number of compounds, possible intermediates between tryptophan and indole, into the latter compound—findings which were in agreement with those previously made by Woods (1935), using washed, viable suspensions of the organism. We next turned to the well-recognized phenomenon, the inhibition of indole production by cultures of *B. coli* in the presence of fermentable carbohydrates. Frieber (1921) has suggested that the breakdown of tryptophan to indole takes place in two stages: (1) the formation of indoleacetic acid, and (2) the breakdown of this compound to indole. In the presence of fermenting carbohydrates, the breakdown of tryptophan proceeds as far as indoleacetic acid, but no further. This hypothesis seemed extremely unlikely to us, not only because of the work of Woods (1935), but also because of our repeated failure to discover indoleacetic acid in glucose broth cultures of normal strains of *B. coli*.

In the experiments which follow, it should be understood that the enzymes were generally prepared from 24-hour cultures on ordinary nutrient agar, although we now recognize that much more active preparations are obtained from nutrient agar with a basis of tryptic digest of casein. The methods of preparation were those previously reported. All preparations were tested for sterility and found to be sterile. Tryptophan was estimated quantitatively by a modification of the method of May and Rose (1922), described by Woods (1935). The method was adapted to our own requirements by first extracting the indole with light petroleum in place of ether. The indole was then estimated in the combined petroleum extracts by the method of Happold and Hoyle (1934), tryptophan being estimated in the indole-free residue.

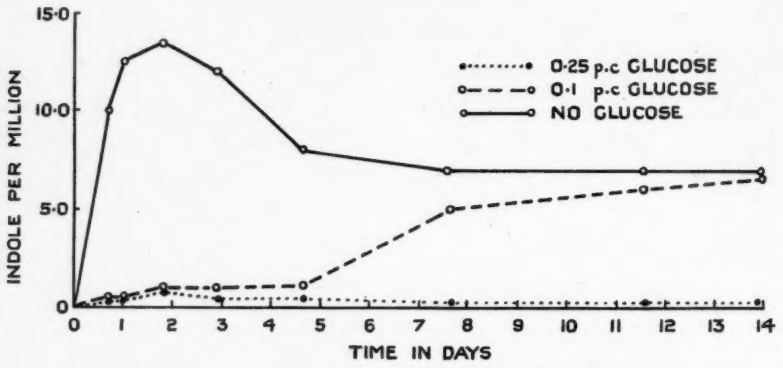
EXPERIMENTAL.

The first fact which was established was that the tryptophanase preparations would not of themselves ferment glucose, and the presence of glucose was without demonstrable effect upon the ability of the enzyme to catalyse the production of indole. Attention was next directed to the relationship between the onset of indole production in glucose broth cultures of *B. coli* and the completion of the fermentation of the carbohydrate. It has long been recognized that the inhibition of indole production in such cultures was not merely a reaction phenomenon, and in the following experiments we have attempted to show this, and also that where the reaction is maintained between pH 6·5 and pH 8·0, by the periodic addition of alkali, indole formation only commences when all the fermentable carbohydrate is utilized.

Absence of Indole Production whilst Glucose is being Fermented.

Three flasks were set up containing Fränkel's synthetic medium and 1 in 40,000 tryptophan. Glucose was added to flasks 1 and 2 in concentration of 0·25 p.c. and 0·1 p.c. respectively, while flask 3 received no glucose. The flasks were inoculated with *B. coli*, incubated, and indole estimated at intervals, the pH measured, and the flasks tested for sugar. The result is shown in Fig. 1.

In flask 1, containing 0·25 p.c. glucose, the reaction became so acid as to practically sterilize the culture, the sugar was not completely utilized in the duration of the experiment, the reaction remained acid, and apart from a very minute initial indole production no indole was formed. In flask 2, containing 0·1 p.c. glucose, only a very minute trace of indole was produced in the first 5 days. At the end of this time the sugar was completely utilized, the reaction reverted to the alkaline side, and coincident with the change of reaction, indole production took place. In the control flask 3, containing no sugar, rapid indole production occurred; the tryptophan present was almost quantitatively converted to indole in 48 hours; on further incubation the indole content fell to about 50 p.c.



EFFECT OF GLUCOSE ON INDOLE PRODUCTION BY B.COLI

FIG. 1.

Similarly, indole production was measured in two parallel cultures of *B. coli*, one in ordinary broth and one in 1 p.c. glucose broth, the reaction of the glucose broth culture being adjusted to pH 8·0 at frequent intervals by addition of alkali. The result is shown in Fig. 2. It will be seen that in spite of the control of the reaction, no appreciable indole production occurred in the glucose broth culture until all the sugar had been used up, while in the control flask, rapid indole production occurred. The inhibitory effect of glucose is, therefore, not due merely to the development of an acid reaction.

Absence of Indole Derivatives whilst Glucose is being Fermented.

The possibility which now suggested itself to us was that in the presence of fermenting carbohydrate semi-anaerobic conditions were set up, with a resultant diversion of the reaction to the formation of indolepropionic acid. That this is not so appears from the following: A flask of Fränkel's medium and 1 in 10,000 tryptophan and 0·5 p.c. glucose was inoculated with *B. coli* and incubated. After 24 hours the culture was acid, no indole was present, and there was no detectable decrease in the tryptophan content.

250 ml. of the culture was boiled, shaken with kieselguhr and filtered. The filtrate was acidified and extracted with ether. The ethereal extract was concentrated over 2 p.c. sodium carbonate and finally extracted with 2 p.c. sodium carbonate; the extract was neutralized and tested for indole derivatives. No indolepropionic acid, indoleacetic acid or other derivative was found. The procedure was repeated at 48 hours and subsequently, with the same results. No evidence was therefore obtained to suggest that glucose diverted the course of the breakdown. It should be noted not only that there is an

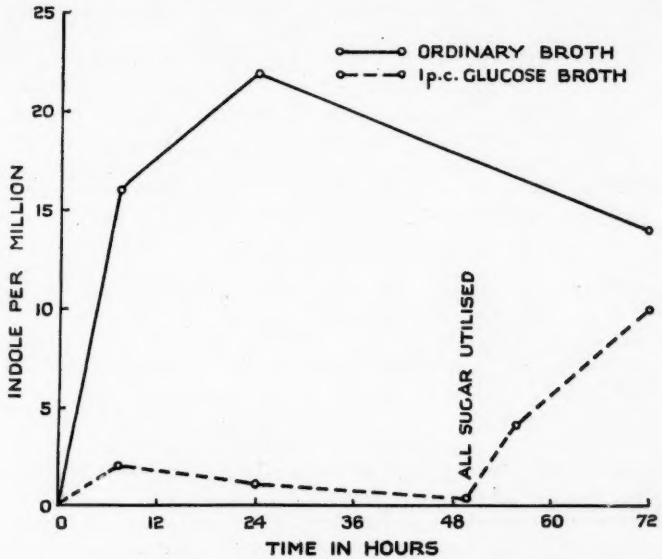


FIG. 2.

alternative source of nitrogen present in the medium and that the fermentation sterilizes the culture, but that if the pH of the culture is adjusted and the experiment prolonged, the concentration of tryptophan falls.

It was next decided to determine if cultures of *B. coli* on glucose agar contained tryptophanase. The organism was grown on large Roux bottles of ordinary agar, and on bottles of 0·5 p.c. glucose agar. Chloroform-killed enzyme preparations were made and then tested for action on 5 mg. of tryptophan in phosphate buffer pH 8·4 at 37° C. under toluene for 48 hours with the following result: enzyme from 12 Roux bottles of ordinary agar yielded 0·6 mg. indole; enzyme from 12 Roux bottles of glucose agar produced no indole.

Further, the enzyme from 5 Roux bottles of agar solidified with a tryptic

digest of caseinogen produced 0·84 mg. indole from 2 mg. tryptophan. When glucose was incorporated in the medium the resultant suspensions from 5 and 10 Roux bottles were separately without demonstrable action.

The cultures on glucose agar appeared therefore to contain no tryptophanase. The experiment was repeated with the difference that dried enzyme preparations were made instead of chloroform-killed preparations. A similar result was obtained: dried enzyme from 12 Roux bottles of ordinary agar yielded 0·13 mg. of indole from 2 mg. of tryptophan; dried enzyme from 12 Roux bottles of glucose agar produced no indole.

These experiments have been repeated several times, but in no case has tryptophanase been detected in cultures from media containing glucose.

Three explanations are possible for this result. Either the organism does not elaborate tryptophanase when growing on media containing sugar, or the tryptophanase is destroyed by some product of glucose fermentation, or forms some semi-stable compound with intermediates or products of the glucose fermentation. It was found that enzyme preparations from sugar-free media produced indole from tryptophan both in the presence and absence of glucose, but that the enzyme did not itself ferment the sugar.

Products of Glucose Fermentation by B. coli *do not Inhibit Tryptophanase Action.*

The following experiment was devised to test whether tryptophanase would produce indole from tryptophan even in the presence of fermenting sugar. 1 mg. of tryptophan was added to 20 ml. of 0·5 p.c. glucose broth, the flask inoculated with *B. coli* and allowed to grow at room temperature overnight. Chloroform-killed enzyme preparation from 8 Roux bottles of ordinary agar was then added and the flask aerated at 37° C. for 48 hours, the reaction being adjusted to pH 8·4 at intervals. Change of reaction throughout the experiment indicated that fermentation of the glucose was taking place, and a subculture at the end of the experiment gave a heavy growth of *B. coli*, nevertheless 0·05 mg. of indole was produced.

The experiment was repeated with a dried enzyme preparation and again indole was produced. It was therefore concluded that neither glucose nor its products of fermentation would inactivate tryptophanase.

It is obvious that a proper control of this experiment is not possible. The metabolism of the carbohydrate by the living *B. coli* causes a constant moving of the reaction to the acid side, and although the reaction is adjusted at intervals, the enzyme is not working at its optimum reaction throughout the experiment. It is not therefore possible to compare the yield of indole with the yield which would be produced by the same quantity of enzyme in the absence of fermenting sugar, since it is impossible to produce parallel variations of reaction in a control flask. It was evident that the products of glucose fermentation did not totally inactivate the added tryptophanase, but it was not possible to say that partial inhibition of the reaction did not occur. With a view to testing this latter possibility, the following experiments were done.

B. coli was grown for 24 hours at 37° C. in Fränkel's medium + 1 p.c. glucose, and the culture sterilized by Seitz filtration. Small quantities of

enzyme were then added to large amounts of the sterile filtrate (50 ml. and 100 ml.) and allowed to stand for 1 hour in the aeration flasks, and then 1 mg. of tryptophan was added to each flask and the flasks aerated. The reactions were adjusted to pH 8·5. Parallel controls were set up in which the filtrate was replaced by saline. In every case the indole production in the presence of the filtrate was greater than in the corresponding control. It is therefore evident that the products of glucose fermentation exerted no inhibitive action on tryptophanase—in fact they appeared for some unknown reason to facilitate its action.

We therefore conclude that in the presence of fermentable carbohydrate the organism does not produce tryptophanase, and that herein lies the explanation of the failure of the bacillus to produce indole under such cultural conditions.

It appears probable that the carbohydrate affords a readier source of energy for *B. coli* than does the tryptophan, so that the bacillus does not require to elaborate a mechanism which it has evolved for normal life. Kendall and his collaborators have previously suggested that the addition of fermentable carbohydrates to cultures of bacteria resulted in a " protein-sparing action " by the carbohydrates, because they afforded a source of energy which was easily available. This work of Kendall's has not been accepted by all workers, and it should be made clear that in the present work we are concerned with an oxidative change ; indeed such evidence as we possess supports the view that the normal deamination of amino-acids by cultures of *B. coli* is not retarded by a simultaneous fermentation of glucose.

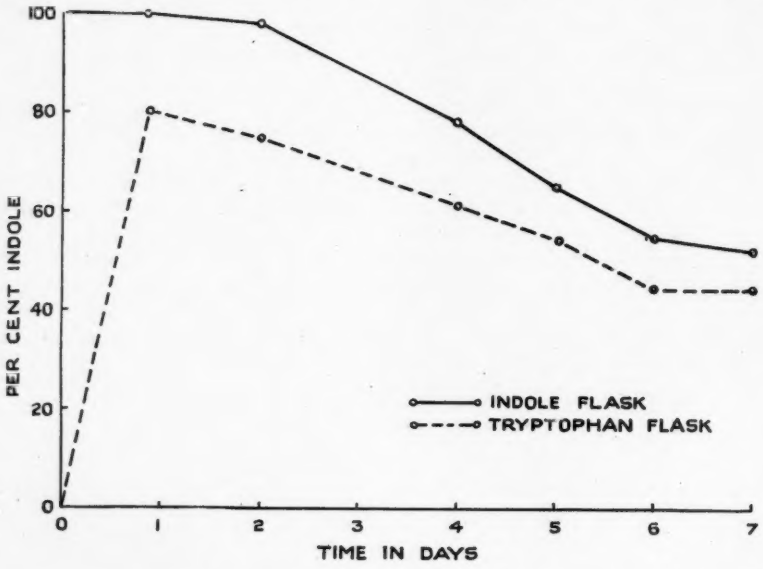
Utilization of Indole by B. coli.

As previously reported by the authors, tryptophanase is produced in cultures of *B. coli* in the absence of tryptophan. It has also been shown by Fildes and Knight (1933) that all organisms which do not require the presence of tryptophan in media to support their growth are capable of effecting its synthesis.

If we refer back for a moment to the first experiment, it will be noted that the final concentration of indole attained by flasks 2 and 3 was the same. There was a marked fall in the indole content of the flask 3 after 48 hours. This fall is a constantly observed phenomenon, and is due not simply to loss by evaporation, but to actual utilization of the indole nucleus. This is shown by the following experiment : Two flasks of Fränkel's medium were set up, one flask containing 1 in 50,000 indole, and the other a concentration of tryptophan equivalent to 1 in 50,000 indole. The flasks were inoculated with *B. coli*, incubated, and the indole content estimated periodically. The loss by evaporation was controlled by estimating the indole against a control flask of medium containing 1 in 50,000 indole which was incubated in parallel with the others. The actual loss by evaporation in this flask was measured, and found to be 20 p.c. in 6 days. The result is shown in Fig. 3. In the flask containing indole and inoculated with *B. coli* the concentration fell to 50 p.c. in 6 days. Indole was rapidly formed in the flask containing tryptophan, attaining 80 p.c. of the possible yield in 24 hours ; it subsequently fell to 45 p.c. in

6 days. In a second experiment with indole added to 1 : 50,000 and 1 : 100,000 respectively, the following results were obtained after 6 days :

	1. Per litre.	2. Per litre.
Initial concentration of indole . . .	0·0200 g.	0·0100 g.
Concentration in uninoculated control .	0·0110 g.	0·0070 g.
Concentration in culture	0·0061 g.	0·0009 g.
Utilization by culture	0·0049 g.	0·0061 g.



UTILISATION OF INDOLE BY B. COLI

FIG. 3.

It would thus appear that in all subsequent experiments where *B. coli* is growing in the presence of indole we must allow for the utilization of a part by the growing organisms, and it may well be that cultures of *B. coli* possessing a tryptophanase system find it easier to synthesize their tryptophan starting with the indole molecule than from the simpler sources of carbon and nitrogen found in Fränkel's medium. This is not incompatible with the 80–100 p.c. production of indole in the early stages of active growth.

SUMMARY AND CONCLUSION.

1. The failure to produce indole by cultures of *B. coli* in the presence of fermentable carbohydrate (glucose) is due to the non-production of a tryptophanase system.

2. Cultures of *B. coli* grown on Fränkel's medium cause a disappearance of indole.

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THE AGGLUTINOGENS M AND N IN ALLERGIC CONDITIONS.

DAVID HARLEY,
Asthma Research Council Fellow.
From the Laboratories of the Inoculation Department, St. Mary's Hospital, London.

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IN 1928 Landsteiner and Levine described two new agglutinable factors, "M" and "N", in human blood-corpuscles, which defined three types of blood, namely, type M (M+N−), type N (M−N+), and type MN (M+N+). These agglutinogens, demonstrated by means of immune rabbit sera, were found to occur independently of the agglutinogens A and B, which determine the four classical blood groups. The agglutinogens M and N were shown to be inherited as Mendelian dominants, dependent on a single pair of allelomorphic genes M and N, there being three possible genotypes, MN, NN and MN, corresponding to the phenotypes M, N and MN. In no instance was a blood found lacking both agglutinogens.

The present communication reports the M and N typing of 200 cases of asthma and allied conditions.

Methods.

Preparation and purification of anti-M and anti-N sera.—Following the technique of Landsteiner and Levine (1928), rabbits were given one intravenous injection of 3 c.c. of fresh washed citrated blood of group O, type M (or N), followed by three intraperitoneal injections of 4 c.c. at weekly intervals. As the response of the animals to this treatment was not satisfactory, they were rested for two months and a second course of three weekly injections of 3 c.c. was given by the intravenous route. The rabbits were bled 7 days after the last injection, the sera collected and stored at −5° C. in 1 c.c. ampoules, without the addition of any preservative. Samples of the "raw" anti-M (anti-N) sera were purified by diluting 1 in 10 with saline and absorbing with half their volume of mixed washed corpuscles of groups A, B and O, of type N (type M), for 45 minutes. With these particular sera a second absorption with

a quarter volume of corpuscles for 30 minutes was necessary to remove completely the species agglutinins. The absorption of the anti-M sera was carried out at room temperature; that of the anti-N at 40° C. The absorbed sera were put up in 1 c.c. ampoules, with the addition of 0·5 p.c. phenol, and were stored in the ice-chest. The specific anti-M and anti-N titres of the purified test sera were approximately 1–16 and 1–32 respectively, with sensitive corpuscles.

The tests.—In addition to the above test sera, commercial anti-M and anti-N sera of continental manufacture were employed. All sera were further diluted 1–2 with saline immediately before the tests. Unit volumes (approximately 6 to 8 c.mm.) of sera were mixed on glass microscopic slides with unit volumes of 2 p.c. suspension of the corpuscles to be tested, by means of a fine throttled capillary pipette. The preparations were placed in moist chambers (Harley, 1936) at room temperature for 30 minutes. The slides were then gently rocked, allowed to stand for 10 minutes, and the reactions examined microscopically with a 2/3 in. objective. Control bloods of known type were included in each batch of tests.

Results.

The frequencies of the three types M, N and MN in the 200 allergic individuals are shown in Table I. There are no published data available concerning the frequencies of the types in the general population in this country, but it will be seen that the figures of the present series correspond closely to those of continental populations.

TABLE I.

	Frequency of types.		
	M.	N.	MN.
Present series . . .	32·00	19·50	48·50
*Schiff (Germans) .	30·94	19·62	49·44
*Crome (Germans) . .	32·50	18·50	49·00
*Kossovitch (French) . .	33·00	21·20	45·80

* Quoted from Wiener (1935).

Wiener (1935) has shown that if a given population is homogeneous with respect to M and N, the sum of the square roots of the frequencies of types M and N should equal unity, on the basis of the Landsteiner and Levine theory of a single pair of allelomorphic genes. If the frequencies are calculated as percentages the sum would be 100. Applying this equation to the present series we get—

$$\sqrt{M} = 56{\cdot}6 \text{ p.c.}$$
$$\sqrt{N} = 44{\cdot}2 \text{ p.c.}$$
$$\sqrt{M} + \sqrt{N} = 100{\cdot}8 \text{ p.c.}$$

As the probable error of the deviation for a population of 200 is $\pm$ 2·4 (according to Wiener) and the actual deviation is + 0·8, the "fit" is good.

There is no indication therefore that the frequencies of the types M, N and MN in the present group of allergic individuals differ from those in the general population.

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A FALSE AGGLUTINATION REACTION OBSERVED WITH ANTI-M AND ANTI-N SERA.

DAVID HARLEY,
Asthma Research Council Fellow.

From the Laboratories of the Inoculation Department, St. Mary's Hospital, London.

Received for publication February 20th, 1936.

IN the course of the preceding investigation an unusual type of agglutination was observed. When certain concentrations of anti-M serum were mixed with a 2 p.c. suspension of human corpuscles lacking the M agglutinogen (or anti-N serum with corpuscles lacking the N agglutinogen) on glass slides and gently rocked, the corpuscles often agglutinated into definite clumps which appeared to differ from the type-specific M and N agglutination only in degree. When the preparation was allowed to remain at rest these clumps gradually disintegrated and the corpuscles finally became evenly distributed (see Plate). The process could be repeated by again rocking the slide. The speed of the disintegration of the clumps could be greatly increased by firmly holding one end of the slide and gently " flicking " the other end with the finger-nail. On examining such clumps with the 1/6-in. objective the corpuscles were seen to be more loosely packed together than is the case with clumps of specific M and N agglutination.

This phenomenon was observed with sera which had been diluted to 1 in 10 for absorption, but which had not been diluted further for the tests. When these sera were further diluted to 1 : 20 or 1 : 30 they failed to produce the reaction. The reaction was independent of the group of the corpuscles employed, though there were variations in the intensity of the reaction with suspensions of different corpuscles. Individual sera differed in their capacity to elicit the reaction, but it was found rather more frequently with a brand of commercial sera than with the writer's own preparations. A number of normal rabbit sera were tested, and though the majority of these gave the reaction in dilutions of 1 : 8 to 1 : 16, it was not so marked as that produced by the anti-M and anti-N sera. Absorption of an anti-N serum with 1/5 its volume of M corpuscles did not appreciably diminish the intensity of the reaction with the corpuscles used for the absorption.

The reaction would appear to be due rather to some non-specific physical property of rabbit serum than to traces of species or other agglutinins resulting from incomplete absorption. Polayes, Lederer and Wiener (1929) observed a non-specific false agglutination similar to the above when testing sera from the umbilical cord of infants with A and B corpuscles; they stated that the reaction was due to the Wharton's jelly with which the sera were contaminated.

Whatever may be the explanation of the non-specific false agglutination reaction between rabbit anti-M and anti-N sera and human corpuscles, its bearing on the practice of M and N typing by the slide method is obvious:

1. Anti-M and anti-N sera should be diluted more than 1 in 10, before or after absorption, for test purposes.
2. Doubtful reactions should be re-examined after the slide has been allowed to remain at rest for 10 minutes.
3. Preferably each corpuscle suspension should be put up with a series of dilutions of the test sera.

When these safeguards were employed this false agglutination reaction did not interfere with the specific reactions in M and N typing.

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THE EFFECTS OF EXHAUST GASES FROM INTERNAL COMBUSTION ENGINES AND OF TOBACCO SMOKE UPON MICE, WITH SPECIAL REFERENCE TO INCIDENCE OF TUMOURS OF THE LUNG.

J. ARGYLL CAMPBELL.

From the National Institute for Medical Research, Hampstead.

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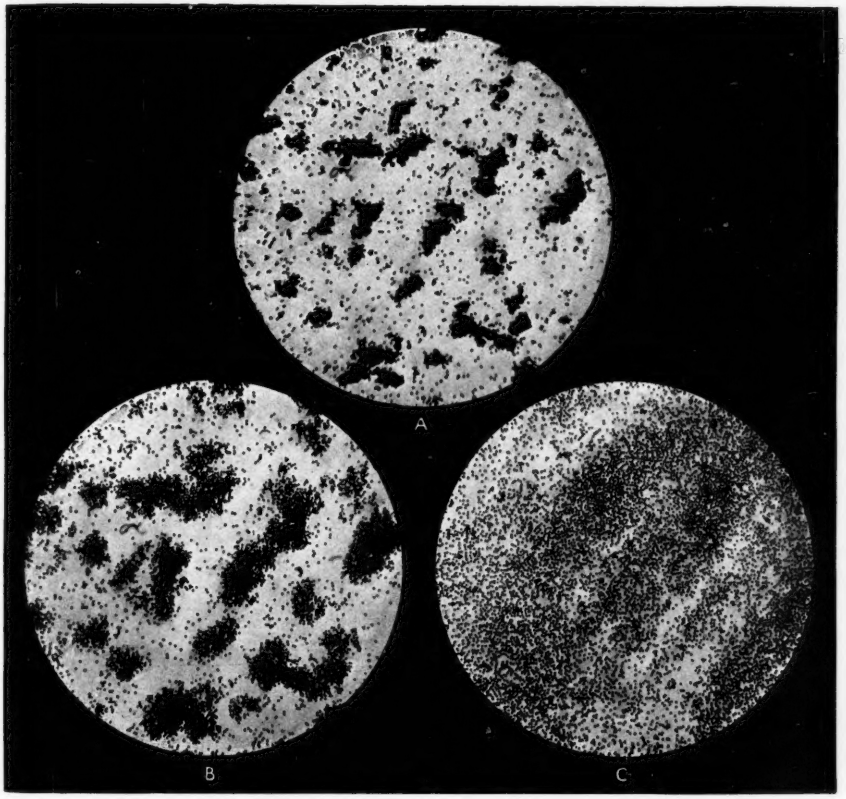
A GOOD deal of attention is being directed at present to the question whether lung tumours are increasing in incidence in man. Most of the evidence is in favour of such an increase, but whereas in some hospitals and countries the increase appears to be striking, in others no such increase has been detected. Tar dust, exhaust fumes and tobacco smoke have been suspected as causes. The use of motor vehicles and the consumption of cigarettes have increased enormously in recent years.

We are considering here only the experimental aspect of the problem. There is definite evidence from several observers that tar and other carcinogenic agents increase the incidence of primary lung tumours in mice. Thus Murphy and Sturm (1925) in some tar-painting experiments found that about 80 p.c. of the mice developed primary lung tumours compared with only 5 p.c. of the controls; the painting was carried out in such a way that skin cancer was avoided. Schabad (1928, 1934, 1935) obtained similar results with



False agglutination with anti-N serum and human blood-corpuscles lacking the N agglutinogen.

Equal volumes of anti-N serum (commercial), undiluted, and 2 p.c. suspension of corpuscles lacking the N agglutinogen, on a glass slide:

A. Immediately after rocking the slide for 2 minutes.

B. Same preparation after remaining at rest for 5 minutes.

C. Same preparation 30 seconds later, the end of the glass slide having been gently "flicked" with the finger-nail.

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tar and also with 1 : 2 : 5 : 6-dibenzanthracene injected subcutaneously; with the latter an increase in lung tumours was noted also by Andervont (1935). The present author (Campbell, 1934*a*) observed that prolonged exposure of mice to dust collected from tarred roads causes cancer of the skin and greatly increases the incidence of primary lung tumours; in this particular experiment at least 20 p.c. of the tumours were of the malignant type, with cancerous cells.

In the present paper the effects on mice of the breathing for prolonged periods of air contaminated with gases from exhaust pipes of internal combustion engines or with smoke from tobacco are investigated. Sayers and his co-workers (1927) published the result of an extensive and very careful study of exposure of monkeys, dogs, rabbits, guinea-pigs and pigeons to such atmospheres polluted with exhaust gases for periods covering several months. They concluded that the impurities, including lead from tetra-ethyl lead, were not present under ordinary circumstances in concentrations likely to affect the public health. The present paper extends the work of these observers in that mice were exposed for periods covering the greater part of their lives—that is, for 7 hours on 5 days every week from the age of about 3 months until their death. The advantage of using mice is that experiments to cover their whole lives last about two years—a much shorter period than if any of the above animals were employed; also mice are subject to a primary lung tumour which appears suitable for experimental investigation of its incidence. Again, any results obtained for factors operating during the whole life of a mouse might be applied qualitatively to similar factors operating through the whole life of a man. Man, in modern cities, breathes continually a more or less polluted atmosphere. The time-factor may be important, as in certain other types of cancer where very prolonged periods of exposure to the "irritant" are necessary.

GENERAL METHODS.

The exhaust gases were collected from two motor vehicles belonging to this Institute. One of them, car A, consumed an ordinary type of petrol, and the other, car B, a blend of petrol with tetra-ethyl lead in the proportion of almost 1 in 1800. Car A was used for Exp. A, car B for Exp. B. The necessary amount of exhaust gas was added to the air in a respiratory chamber to produce the desired concentration of carbon monoxide. There was no through ventilation of air in the chamber; the latter was closed and the air in it kept well mixed by means of a fan fixed at the top and driven by a motor outside the chamber. The air-space of the chamber was, in each case, about 580 litres (89 × 77 × 85 c.c.). About 75 mice, 3 months old, were placed on the floor in boxes with freely perforated lids. It was not found necessary to absorb the carbon dioxide or the moisture produced by the mice, and the former did not exceed 3 p.c. in the air of the chamber at the end of 6 hours. The mice used only a small amount of oxygen, about 1 c.c. per mouse per minute, and the concentration of oxygen in the air of the chamber did not fall further from 20·9 p.c. than to 17 in 3 hours, at which time an extra 30 litres of oxygen were added. Mice show no evidence of adverse effect when breathing 3 p.c. carbon dioxide and 17 p.c. oxygen, and the variations in the present case were from about 0 to 3 p.c. carbon dioxide and from 20·9 to 17 p.c.

oxygen. It was thought advisable to allow these variations rather than to place soda lime in the chambers, which might absorb some of the other exhaust gases. Further, an increase of carbon dioxide and decrease of oxygen are characteristic of exhaust gas. A through ventilation of air containing a fixed percentage of exhaust gas might have been nearer the ideal, but the results very likely would not have been different, and the expense and attention needed for three separate through ventilation systems would have been very much greater. A mouse produces about 1 c.c. of carbon dioxide per minute and the conditions in a large respiratory chamber dilute this. This combination of facts greatly simplified and cheapened the research.

The mice were placed in the chamber from 10 a.m. to 5 p.m. every day of the week except Saturday and Sunday. During this period in the chamber they were supplied with water and sawdust, but no food. Food was placed in other boxes used from 5 p.m. on one day until 10 a.m. on the next day. Controls were fed with similar food (wheat, barley, oats, greenstuff, wholemeal bread soaked in water and rolled oats).

A similar routine and chamber were used for Exp. C, in which mice were exposed to smoke from cigarette tobacco.

For analyses of the undiluted gases an Orsat type of apparatus (Burrell and co-workers, 1926) gave results with accuracy to the first decimal place for a mixture of gases containing carbon dioxide, unsaturated hydrocarbons or illuminants, oxygen, hydrogen, carbon monoxide, methane, ethane and nitrogen. This method was slower and less accurate when the carbon monoxide was present in low concentration, *e. g.* 1 p.c. ; the copper oxide for combustion of the carbon monoxide seemed more efficient with higher concentrations and also when heated to 350° C. The above authors recommended 290°–310° C. but their laboratory may have been warmer than ours (15–20° C.), so that their gas would be more rapidly heated up. We found that Haldane's large apparatus, with combustion pipette, often gave fairly accurate results for carbon monoxide, because other combustible gases were present on these occasions in small quantities only.

The main control for the experiments was the accurate iodine pentoxide method for estimating 0·01 p.c. or 1 in 10,000 carbon monoxide. In some few cases Hartridge's spectroscope and the blood method were employed, but the iodine pentoxide method on the lines recently described by Haldane and Graham (1935) was found the more satisfactory under our conditions. The iodine pentoxide was prepared by the British Drug Houses from iodine and chloric acid and gave reliable results with controls. The above American workers among others found this method of preparation very satisfactory.

EXPERIMENT A : EXHAUST GASES FROM COMBUSTION OF AN ORDINARY PETROL.

The exhaust gas as collected from car A usually contained about 5 to 9 p.c. of carbon monoxide. Frequent analyses were made and some examples of results are given in Table I.

The four cylinders of this car were 3·676 in. diameter and 4·25 in. stroke, while the horse-power was about 23. When running on the road the

TABLE I.—*Analyses (p.c.) of Undiluted Exhaust Gas from Car A, using Ordinary Petrol.*

Date.	Method.	CO_2.	Ills.*	O_2.	H_2.	CO.	CH_4.	C_2H_6.
28.iii.34	Haldane	9·1	..	2·8	0·8	4·7	0·9	..
7.vi.34	,,	7·2	..	3·2	1·7	7·3	2·4	..
10.i.35	,,	8·9	..	2·5	1·6	6·4	1·3	..
25.i.35	,,	9·6	..	0·5	2·9	8·5	0·0	..
24.vii.35	,,	7·8	..	3·1	3·3	7·7	0·8	..
24.ix.35	Orsat	7·1	1·6	3·2	3·4	7·1	0·5	0·0
30.ix.35	,,	6·1	0·9	4·7	3·7	7·6	0·2	0·4
4.x.35	,,	6·8	0·6	1·2	5·0	9·7	0·1	0·6
8.i.36	,,	7·0	0·4	2·4	4·8	9·4	0·6	0·2
10.i.36	,,	8·0	0·4	2·2	4·2	6·8	0·6	0·0

* Unsaturated hydrocarbons or illuminants.

engine consumed 1 gallon of petrol for 25 miles. In the experiment the engine was allowed to run for 5 minutes with the car stationary, and then the exhaust gases were collected in a bag of 60 litre capacity. The petrol used was only about 25 c.c. per minute and the petrol-air ratio was 1 : 10. A commonly-used lubricating oil was employed, and some skin-painting tests with this oil have produced warts in 2 out of 26 mice still under investigation.

The experiment was commenced on December 11th, 1933, with 75 mice, 38 of which were females and 37 males. The concentration of exhaust gas in the chamber was gradually increased, so that at first the mice were breathing almost 0·01 to 0·02 p.c. carbon monoxide, but for the last 657 of the total 753 days of exposure the exhaust gas was diluted 1 in 145 from 10 a.m. to 2 p.m., and 1 in 83 from 2 p.m. to 5 p.m. Thus if the exhaust gas contained 6 p.c. of carbon monoxide, the mice in the chamber breathed 0·041 p.c. in the morning and 0·072 p.c. in the afternoon. Some examples of accurate analyses are given in Table II. The concentrations of the other exhaust gases in the chamber were, apart from oxygen and carbon dioxide, less than that for carbon monoxide; they may be estimated by dividing the figures in Table I by 83. Very little exhaust carbon (soot) was present in the chamber; this was estimated by collection in small glass trays. Such carbon may be impregnated with oil and other matter, and sooner or later will be deposited on the roads. It must have been included in the previous research (Campbell, 1934*a*) with the dust from tarred roads. Separate experiments with mice breathing air contaminated with such soot are contemplated.

TABLE II.—*Analyses of Chamber A Gas containing Diluted Exhaust Gas from Car A.* I_2O_5 *Method for CO p.c.*

Date.	CO p.c.	
	a.m.	p.m.
26.vii.35	0·031	0·067
2.viii.35	0·046	0·084
30.ix.35	0·040	..
4.x.35	0·052	..
1.xi.35	0·020	..

The average concentration of carbon monoxide throughout most of the experiment was slightly below 0·05 during the morning and somewhat above this during the afternoon. In traffic blocks the concentration found is about 0·01 p.c., but the higher concentrations were used for the mice, because mouse's blood is less saturated by a given concentration of carbon monoxide than is human blood. Also by using the higher concentration any disadvantage of not using a through ventilation would be counter-balanced. On some few occasions the concentration of carbon monoxide breathed by the mice was about 0·1 p.c., but it has been proved (Campbell, 1934*b*) that mice after gradual acclimatization can grow normally, although losing fertility when breathing 0·2 to 0·3 p.c. of the pure gas. This concentration, 0·25 p.c., has a retarding effect upon cancer growth in mice (Campbell, 1932, 1933); nevertheless it was thought advisable to test all exhaust gases together as they occur in traffic blocks or garages. The main details of the results are given

TABLE III.—*Exp. A: Mice Exposed to Diluted Exhaust Gas from Car A.*

Date.	Number of days of experiment.	Number of mice alive.		Weight of living mice (g.).		Animals with lung tumours.	
		Control.	Experiment.	Control.	Experiment.	Control.	Experiment.
5.xii.33	0	74	75	15·6	15·4	..	..
15.iii.34	100	67	66	22·6	21·1	..	..
23.vi.34	200	58	49	26·3	25·9	..	..
1.x.34	300	58	41	29·5	29·6	..	1
9.i.35	400	45	31	29·6	29·8	..	1
19.iv.35	500	24	18	29·8	30·0	2	4
28.vii.35	600	18	11	28·2	30·1	3	5
5.xi.35	700	6	7	28·5	29·7	7	7
3.i.36	759	4	2	27·2	25·5	8	9

in Table III. There was no material difference in death-rate, body-weight and rate of growth between the experimental mice and the controls in all three experiments A, B and C. The death-rate is more rapid than that of the controls in Exp. A, but not than that of the controls in Exp. B (see Table VI). There is scarcely any difference in the incidence of primary tumours of the lung. Thus in Exp. A there were 8 control mice and 9 experimental mice with such tumours. Most other pathological changes were fairly equally divided between the experimental animals and the controls. Three of the controls and 2 of the experimental mice showed mammary tumours. One of the controls possessed a tumour of the liver, whilst amongst the experimental mice there was one with a tumour near the lower end of the œsophagus and cardiac end of the stomach and one with a tumour of the skin near the lower jaw. Metastases were rare; this has seemed to be the case always with our mice. The commonest cause of death seemed to be congestion of the lungs due to heart failure. Advanced stages of pneumonia were observed in 10 of the experimental mice and in 2 of the controls. Liver changes included the usual parasitic cysts; congestion and also atrophy with fibrosis or necrosis were observed in 7 of the controls and 4 of the experimental mice. Further

fertility was not affected by the concentration of the gases used; about 70 p.c. of the 20 females tested were fertile as in the controls.

EXPERIMENT B: EXHAUST GASES FROM COMBUSTION OF A PETROL CONTAINING TETRA-ETHYL LEAD.

Car B burning this petrol had six cylinders, each of 500 c.c. capacity, while the horse-power was 24. On the road the car used 1 gallon in 15 miles. With the car stationary the engine, as run in the experiment, consumed only about 40 c.c. of petrol per minute, and the petrol-air ratio was about 1 : 11. Only harmless quantities of lead could have been present in the air in the chamber. Legge and others (1912) stated that 0·5 mg. of lead per cubic meter or 0·014 mg. per cubic foot of air was the lowest dose that would eventually produce lead-poisoning after years of exposure; in this calculation a daily exposure of 10 hours was assumed. Sayers and his co-workers (1927) found that this was true also for their animals exposed to gasolene containing tetra-ethyl lead. The conditions in our present experiment were less likely to cause ill-effects than Sayers Campaign I experiment, in which no lead symptoms were observed. The dust deposited from the chamber air was collected in small glass trays and tested for lead. Its presence could not be demonstrated.

Table IV gives some examples of the analyses of the undiluted exhaust gases. These agree with the analyses of Sayers and co-workers for exhaust gases from an engine burning ethyl gasoline under similar conditions.

TABLE IV.—*Analyses (p.c.) of Undiluted Exhaust Gas from Car B, using Ethyl Petrol.*

Date.	Method.	CO_2.	Ills.	O_2.	H_2.	CO.	CH_4.	C_2H_6.
12.vi.34	Haldane	9·0	..	3·5	0·0	3·9	1·5	..
17.i.35	,,	12·9	..	0·9	0·3	2·3	0·3	..
24.vii.35	,,	10·0	..	6·1	0·0	0·5	0·2	..
24.ix.35	Orsat	8·9	0·4	6·6	0·6	1·6	0·0	0·0
30.ix.35	,,	9·2	0·4	5·0	0·7	2·3	0·8	0·0
8.x.35	,,	9·6	0·2	5·6	0·2	2·0	0·0	0·2
7.i.36	,,	10·2	0·2	4·4	1·2	2·4	..	..
9.i.36	,,	9·6	0·2	5·2	1·2	2·2	..	..
10.i.36	,,	10·8	0·2	3·2	0·8	1·6	0·6	0·2

TABLE V.—*Analyses of Chamber B Gas containing Diluted Exhaust Gas from Car B. I_2O_5 Method for CO p.c.*

Date.	CO p.c.	
	a.m.	p.m.
9.viii.35	0·022	0·037
13.viii.35	..	0·013
27.ix.35	..	0·012
4.x.35	..	0·029
22.x.35	..	0·022
8.xi.35	..	0·013
22.xi.35	..	0·017

The diluted exhaust gases were breathed by the mice for 751 days, and on 604 of these the exhaust gas was diluted 1 in 145 during the morning and 1 in 83 during the afternoon. Thus, for the greater part of the experiment, the mice breathed about 0·01 p.c. in the morning and usually below 0·03 p.c. in the afternoon (see Table V). So far as the gases are concerned, the conditions were much less likely to cause ill-effects than those of Exp. A. Table VI gives details showing that the conditions of the experimental mice compared favourably with the controls; thus their death-rate on the whole was somewhat slower, and they maintained body-weight fairly well up to the 400th day, after which they appear to have lost more weight than the controls. This probably is of no significance, since the controls for Exp. C (Table IX) showed a similar loss of weight at the same age. When comparing results it has been considered wiser to make use of all three batches of controls from Exps. A, B and C. All the mice were reared under similar conditions.

TABLE VI.—*Exp. B: Mice Exposed to Diluted Exhaust Gas from Car B.*

Date.	Number of days of experiment.	Number of mice alive.		Weight (g.) of living mice.		Animals with lung tumours.	
		Control.	Experiment.	Control.	Experiment.	Control.	Experiment.
10.i.34	0	70	75	18·7	18·5	..	..
19.iv.34	100	58	63	22·5	23·0	..	..
28.vii.34	200	46	46	26·7	27·7	..	1
5.xi.34	300	38	44	28·3	29·0	1	1
13.ii.35	400	22	34	31·0	30·4	1	1
23.v.35	500	17	26	30·0	28·6	1	4
31.viii.35	600	11	14	31·0	27·8	3	6
9.xii.35	700	4	6	31·0	27·6	4	10
29.i.36	751	3	3	32·0	26·0	6	12

As regards the incidence of primary lung tumours, the experimental mice show an increase over the controls. This increase, however, is probably not great enough to be significant, as will be shown later. Four of the experimental mice and 2 of the controls had mammary tumours; also among the controls there were 2 mice with skin cancers. The other pathological changes resemble those given for the mice in Exp. A and need no special attention, since in these respects the controls and experimental mice resembled one another. Also fertility was not affected adversely, being somewhat higher in the experimental mice than in the controls.

EXPERIMENT C: EXPOSURE TO GASES FROM COMBUSTION OF CIGARETTE TOBACCO.

The cigarettes, a Virginian variety in great demand, were smoked one at a time by means of a Higginson syringe. One end of the cigarette was placed in a short glass tube, 7 mm. in diameter, which was fixed to the upperside of a wider glass tube, 20 mm. in diameter and 16 cm. in length and closed at one end. To the other end the Higginson syringe was attached by means of

tubing 18 cm. in length and 5–7 mm. in diameter. The outlet from the syringe was attached to the lid of the respiratory chamber by a tube 30 cm. in length and 3–5 mm. in diameter. Part of this tube was of glass, with, on its lower side, a small bulb to collect any fluid condensing from the smoke. The sealed end of the glass cigarette holder was placed at a lower level than the end attached to the syringe, so that condensing fluid was also collected here. This fluid was dark brown in colour, and contained some of the tarry matter from the smoke. Much of the tarry matter was blown into the chamber, and was sufficient to stain the coats of the white mice a pale yellow colour. Cardboard flooring was placed under the boxes in the chamber and kept there throughout the experiment. The exposed portions of this absorbed the tarry matter, and at the end of the experiment the whole flooring was stained a very dark brown compared with similar cardboard used in another chamber where such tarry matter was absent. This tarry matter was removed with benzole from the cardboard for estimation. Also some of the tarry matter which entered the chamber was collected in small flat glass dishes ; from this it was estimated that each mouse came into contact with the equivalent of at least 4 mg. of concentrated tar per month—that is, each mouse box (10 mice) received 40 mg. tar per month ; the tarry matter was observed in phagocytes in the lungs. In the tar-dust experiment (Campbell, 1934*a*) each mouse came into contact with much more tar—at least 30 mg. of tar per month.

It is not known whether tobacco tar contains benzypyrene, the carcinogenic agent present in coal tar. We have painted the skin of some mice with this tobacco tar, and so far, like others, have failed to produce any cancerous change, although one mouse is at present showing a suspicious wart-like growth. Lickint (1935) gives some statistical evidence that bronchial cancer may be concerned with tobacco smoking, but, so far as we know, no cancerous agent has yet been obtained from tobacco. Concentrated tobacco tar looks like coal tar.

The nicotine entering the chamber was estimated by means of Pfyl's method (1934). This indicated that for 617 days of the experiment 20 mg. of nicotine from 12 cigarettes were blown daily with the smoke into the chamber. Dixon (1928) states that if a cigarette smoker were to puff 10 cigarettes on end he would certainly absorb 20 to 30 mg. of nicotine. This is enough to produce a profound physiological effect, though administered in this way perhaps 1/10th to 1/15th of the lethal dose for man. It is not known exactly how much nicotine each mouse actually absorbed in the present experiment, but the mice certainly did not show any obvious symptoms.

Each cigarette required about 14 to 15 pumps of the syringe for nearly complete combustion, and about 2 litres of air passed through the cigarette into the chamber. With 6 cigarettes used in the morning the smoke was diluted about 1 in 50 in the chamber, and with another 6 cigarettes smoked into the chamber at 2 p.m. the dilution was reduced to about 1 in 25. Examples of analyses of the smoke before dilution in the chamber are given in Table VII. The figures for carbon monoxide are not regarded as being very accurate. The very accurate limits of concentration of carbon monoxide breathed by the mice are given in Table VIII. If the morning concentration be multiplied by 50 and the afternoon concentration by 25, the concentration of carbon

TABLE VII.—*Cigarette Smoke before Dilution in Chamber; Orsat Apparatus; Percentages of Atmosphere.*

Date.	CO_2.	Ills.	O_2.	H_2.	CO.	CH_4.	C_2H_4.
29.ix.35	4·8	1·1	16·2	0·2	1·1	0·3	0·4
26.ix.35	4·4	1·0	15·9	1·2	1·0	0·0	1·1
13.i.36	7·0	0·5	13·9	1·8	1·2	0·3	1·2
14.i.36	6·3	0·8	14·1	0·8	1·2	0·0	1·2

monoxide in the undiluted smoke will become about 1 to 1·5 p.c. This agrees with the figure given by Armstrong (1922) under similar conditions of quick smoking.

In addition to nicotine, tarry matter, carbon monoxide and the other gases mentioned in Table VII, tobacco smoke contains also ammonia gas and pyridine or pyridine derivatives. In tobacco smoking the only serious effect of these pyridine bodies is local irritation (Dixon, 1928).

TABLE VIII.—*Cigarette Smoke: CO Concentration (p.c.) in Chamber Gas.*

Date.	CO p.c. a.m.	CO p.c. p.m.
25.vii.35	0·025	0·055
6.viii.35	0·031	0·065
13.viii.35	0·033	0·065
26.ix.35	0·028	0·050
18.xi.35	0·023	0·045
25.xi.35	0·030	0·055

In the present experiment the mice, on the per kilo basis, were exposed to conditions resembling at least those of a more than moderate cigarette smoker. Nevertheless there was no effect of any importance upon death-rate, body-weight and rate of growth of mice (Table IX). There were 17 experimental

TABLE IX.—*Exp. C: Mice Exposed to Diluted Cigarette Smoke and Tarry Matter.*

Date.	Number of days of experiment.	Number of mice alive. Control.	Number of mice alive. Experiment.	Weight (g.) of living mice. Control.	Weight (g.) of living mice. Experiment.	Animals with lung tumours. Control.	Animals with lung tumours. Experiment.
14.viii.33	0	76	74	18·5	18·6	..	..
22.xi.33	100	65	67	21·9	22·8	..	..
1.iii.34	200	56	58	24·4	24·2	..	..
9.vi.34	300	45	41	26·1	25·5	..	..
17.ix.34	400	41	37	27·7	27·1	..	1
26.xii.34	500	35	31	29·3	30·7	1	1
4.iv.35	600	28	20	27·4	31·7	1	5
13.vii.35	700	12	10	25·0	29·9	4	9
21.x.35	800	3	5	31·3	29·4	10	12
19.xi.35	829	2	3	35·5	27·0	11	17

mice and 11 control mice with primary tumours of the lung; the increase in the case of the former will be discussed later. Three of the experimental females showed mammary carcinomas. The other pathological changes resemble those given for the mice in Exp. A and are of no obvious significance. Fertility was tested, and there was no change compared with the controls.

DISCUSSION.

It is evident that under the conditions of the experiments the exhaust gases and tobacco smoke did not produce any very marked effect upon general health. There seemed to be some increase in incidence of primary lung tumours as the result of exposure to exhaust gas from the car consuming petrol containing tetra-ethyl lead, and also as the result of exposure to cigarette smoke. These increases may be more apparent than real when the age of the mice is considered, and are insignificant compared with the effects previously obtained with dust from tarred roads. This dust, which contained much more tar than the tobacco smoke, was proved to be carcinogenic, since it caused warts and cancer of the skin; so far we have tested four samples of road tar, and all four contain carcinogenic agents as tested on the skin of the mouse.

TABLE X.—*Comparison of Results for Primary Lung Tumours.*

Condition of mice.	Percentage of last surviving 56 mice with primary lung tumours.	Greatest age of mice (days).
Normal controls A	7	664
,, ,, B	7	705
,, ,, C	7	841
,, ,, D	14	849
,, ,, E	20	919
Exhaust gas A	14	849
,, ,, B	20	841
Cigarette smoke	30	919
Tar dust + CO	47	705
Tar dust	80	664

There were two experiments with tar dust (Campbell, 1934*a*), in one of which—that with the smaller amount of tar—the mice also breathed carbon monoxide. The tar dust alone produced primary lung tumours in 80 p.c., or 45 out of 56 mice surviving after the appearance of the first wart on the skin (Table X). For comparison, therefore, we have taken the last surviving 56 mice in each of the other groups of experimental and control mice and estimated in what percentage of these primary lung tumours eventually developed. It is evident (Table X) that the tar dust was much more effective than the exhaust gases or cigarette smoke. As already stated, other observers have noted an increase in incidence of primary lung tumours in mice the skin of which was painted with tar; here the mice may have inhaled particles of the tar, or the tarring of the skin may have increased indirectly the sensitivity or

reduced the resistance of the cells of the lung to tumour agents. At the moment it is not possible to decide whether the increase in incidence of primary lung tumours observed in the mice exposed to tar dust was due to a direct effect of the tar dust, or an indirect effect from changes induced in the skin. An experiment is being conducted at present to determine whether the dust after removal of the tar by benzole is still effective. This experiment is proceeding and, as expected, the dust freed from tar has not produced cancerous effects upon the skin such as were obtained with the tar present; the results for the lung tumours will not be available for some months, as the mice are not all dead yet.

In Tables III, VI and IX it will be noted that the primary lung tumours did not occur in animals dying before about 200 days of the experiment or about 290 days, or 10 months old, adding 90 days for the age when the experiments commenced. Indeed, the tumours are not numerous until towards the end of the second year. Thus age has an effect on the incidence of these tumours, and this must be allowed for in our argument. Table X records that the oldest mouse in the tar-dust experiment was 664 days; this is less than the age of any of the mice in the other groups, so that age was not responsible for the marked increase in the tar-dust experiment. Murphy and Sturm (1925) proved that age was not responsible for the increase in lung tumours observed by them in their tar-painting experiment.

Why should tumours occur at a late age in normal mice—that is, in mice not exposed to experimentation? This is the question needing answer, as does that concerning the incidence of lung tumours in apparently normal man, where the mean age at death is 55 years. Probably the older lung is more sensitive to the agent inhaled, perhaps in dust. The lungs of apparently normal animals may contain dust as the animals get older. In the older lung the cleansing mechanism may break down, so that the dust is not removed as before.

Heredity must also be considered as a factor. With an inbred albino strain of mice, Bittner (1935) found that in males living 10 months or longer, the primary lung tumour incidence was 55 p.c.; he also found that 64 p.c. of the breeding females which lived a further 4 months or longer developed mammary gland carcinomata. Again, 36 p.c. of the breeding females which had mammary gland tumours also had primary or secondary lung nodules. Such inbreeding evidently renders the mice much more sensitive by some mechanism, and would probably do the same in the case of man. Nevertheless an external agent may still be responsible as the direct stimulus. Our mice, however, which were of a mixed strain and which have been under observation for 10 years, have never shown among controls an incidence of lung tumours above 25 p.c., and usually the average incidence for various batches of 75 mice has been nearer 10 p.c. (Table X). Also the incidence of mammary tumours is low amongst these mice. We conclude that heredity is not the main cause of the increase observed in the tar-dust experiment.

Although suggestive, we do not consider that the incidence of lung tumours in the cigarette smoke experiment is high enough above that for the controls of the same age—919 days—to demand special attention, because the increase is due to mice which survived longer than those of any of the other groups.

There is a good deal of argument whether these lung tumours in mice may be compared with lung tumours in man. There are differences, but they do not appear so important as to lead to the conclusion that the mouse lung tumours are not tumours in the same sense as are human tumours. Most human lung tumours give rise to frequent metastases, whereas mouse lung tumours, although usually multiple, do not. Still there are human lung tumours on record with few or no metastases. Thus Sweany (1935) describes a so-called alveolar cell cancer of the human lung which closely resembles some of the mouse lung tumours; there was only one small metastasis in a hilar lymph-gland. Sweany gives the literature and discusses the origin, which seems to have been somewhere between the lower respiratory bronchiole and the alveoli. The common opinion is that the basal layer of the epithelium of the bronchial passages gives rise to the lung tumours. There is a good deal of literature concerning the mouse lung tumours (Campbell, 1934*a*), and the majority of observers describe a malignant invading type as well as the more simple type. Certainly in our tar-dust experiment at least 20 p.c. of the tumours showed cancer-cells. The nucleus is quite atypical, as is also the arrangement of cells, basement membrane, etc.

The tumours appear to arise sometimes near the pleura and sometimes near a bronchiole. Often there is a collapsed area with "embryonic" or glandular alveolar cells surrounded by a rim of cancerous cells. There were no striking histological differences between the tumours in the control groups and those in the experimental groups. The difference in incidence is the main point under investigation. In the tar-dust experiment it was easy to find dust particles in the region of the tumour.

Again, sometimes these mouse lung tumours do not appear to have much ill-effect upon the mice, but it must be remembered that mice with skin cancers due to tar may remain active for long periods and outlive some apparently normal mice. Such differences as exist between tumours of the lung of the mouse and those in man may probably be due to different reactions in different classes of animals.

SUMMARY.

1. Mice were exposed for 7 hours on 5 days each week during most of their lives to exhaust gases from internal combustion engines, in concentrations somewhat resembling those obtaining in traffic blocks and garages. There were no marked effects upon the well-being of the mice. Carbon (soot) and lead were excluded in these experiments.

2. Mice were similarly exposed to fumes and tarry matter from cigarette smoke without any marked pathological effects.

3. The incidence of primary lung tumours in mice is considered in detail. These tumours are usually rare before the latter half of the second year of life. Exposure to exhaust gases has little effect upon this incidence when compared with the controls. The incidence for the tobacco group of mice is on the high side, but these mice survived longer than the other groups.

4. The incidence is increased by dust collected from tarred roads, as shown previously. Some of these tumours are definitely malignant in appearance. It is not known yet whether the dust freed from tar has any influence; this

question is under examination, and the results should be available in a few months. Age and heredity have an influence on the incidence of these primary lung tumours of mice, but these factors are not responsible for the result with the tar dust. This dust contained much more tar than the tobacco smoke.

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